Health Effects from the Inhalation of Oxidant Air Pollutants as Related to the Immune System

Final Report
California Air Resources Board
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Abstract

The original proposal, for the studies reported here, was to:

a. Investigate the effects of ozone and sulfuric acid aerosol for enhancing the syndrome of extrinsic asthma.

b. Examine the effects of ozone on altering the severity and pathogenesis of influenza.

Mice were chosen as the experimental animal for several reasons. Immunological mechanisms have been more thoroughly studied in mice than in any other species, and much that is known about immune responses in man has come from knowledge obtained as a result of mouse studies. Work was performed in the anticipation of finding distinct links between air pollution and lung diseases. It was intended that data from the studies might contribute toward decision making on pollution levels that are compatible with normal lung function.

Major findings of these studies are:

1. Ozone exposure increases the incidence of allergic lung sensitization to an inhaled allergen. Five extensive experiments were performed. An aerosolized allergen (ovalbumin) was used to mimic the inhalation of environmental allergens such as plant pollen. Significantly greater numbers of animals were allergic in experimental groups where ozone exposure ranged from the high of 0.64 ppm to a low of 0.16 ppm.

2. Sulfuric acid aerosol (concentration of 0.5 and 1.0 mg/m^3) did not have a significant effect for the enhancement of allergy to an inhaled allergen. Three experiments were performed. Animals exposed to sulfate alone were not sensitized to a greater extent than the mice breathing filtered ambient air. It was concluded that the allergic enhancement seen in the combined ozone and sulfate groups could be explained on the basis of the ozone effect. This negative finding with respect to sulfuric acid aerosol further delineated the uniqueness of effects on lung tissue from ozone inhalation.

3. In asthma, antibodies of the IgE class are specifically responsible for the disease. Cells producing IgE are distributed along the respiratory mucous membranes and also in the gas exchange tissue. It was important to correlate the allergic state in living animals with cellular responses to show that synthesis of IgE antibody had been stimulated from contact with the allergen. Combined data from three experiments have shown the correct correlation of cells with the sensitivity levels in the animals.

4. Fatal influenza infections were less frequent in ozone exposed animals (0.4 and 0.64 ppm) than in mice breathing ambient air. This observation was made in four separate experiments. Two factors appear to interplay in producing this interesting effect.

a. Abnormal respiratory membranes develop as a result of 15 days of continuous ozone exposure, and this appeared to change the role of lining epithelium as host cells for influenza virus.

b. Ozone, at the concentrations used, was found to inactivate influenza virus. This effect could diminish the spread of virus within the airways.

Broad interpretation should not be made on the basis of these virus infection results because the ozone concentrations used, and the continuously maintained elevation of ozone, were not in accord with usual environmental conditions. It is necessary to pursue these investigations at lower ozone levels.

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Standard Disclaimer

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Health Effects from the Inhalation of Oxidant Air Pollutants as Related to the Immune System

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Statement of the Problem:

The appropriate stance to take regarding acceptable levels of air pollutants in California is affected by the increasing need to determine what actually happens to mammalian tissue following pollutant exposure. A growing body of epidemiological literature presents positive correlations of lung diseases and reduced pulmonary capacity with the presence of high levels of air pollutants (1,2,3). However, skeptics can question the interpretations of such investigations by pointing to uncontrolled variables, and the fact that positive evidence of tissue changes are not shown by such studies. If air pollutants are shown to adversely affect the respiratory tract by enhancing the likelihood of developing asthma, or by altering the recovery from respiratory infections, this information would have impact on public attitudes toward the control of air pollution. Controlled experimentation on animals offers the means for determining alterations in the immune responses of the respiratory tract as mediated by the presence of air pollutants. Factors studied here, both singly and in combination, were ozone and sulfuric acid aerosol. Ozone has been shown by others to act as a powerful oxidizing agent that produces histologically recognizable damage to the lining epithelial cells of the respiratory tract(4,5). The problems accruing from high ozone levels will persist in the foreseeable future as a by-product from the use of internal combustion engines. The South Coast Air Basin with its dense population is especially subject to ozone exposure.

Predictably the generation of SO_2 will increase in California as the combustion of fuel oil and coal increases from stationary sources(1). This will be affected by the increasing demands for electrical energy at a time when the likelihood of safe nuclear power development may be diminishing. Since much of the SO_2 is converted to sulfates and sulfuric acid in the atmosphere, we worked directly with sulfuric acid aerosols. There is much concern about the role of sulfate aerosols in the aggravation of respiratory diseases(1,2). However, experimental evidence is needed to determine how this may come about.

Immunology offers real promise in determining events set in motion by air pollutants. Increases of immunological activity in the lung are clear

indicators of injury to the tissues. Increases in allergic lung disease (asthma) among a test population of animals following inhalation of air pollutants establishes a cause and effect relationship between the toxic inhalant and enhancement of a specific health problem. Changes in the pattern of immunological defenses of the lung against infecting virus (influenza virus) following exposure to air pollutants offers clear evidence that the susceptibility of lung cells to the virus has been altered.

The immune system is primarily concerned with the body's handling of foreign substances entering the tissues. If the foreign substance provokes an immune response it is referred to as an antigen. For example, in an influenza attack the influenza virus acts as an antigen and the immune responses to the viral antigen are specific antibodies and cells reacting with virus, which lead to its destruction and elimination from the body. A good functional immune system is, therefore, vital to the warding off of infections and the maintenance of a healthy state.

In some situations the immune responses play an undesirable role. An example is human asthma where the inhaled antigen is usually some inert substance, such as dog or cat dander, household dust, plant pollens, etc. The antibody response in this case is detrimental to health since the reaction of the antigen and antibodies in the lung leads to the release of body chemicals that contract the airways and produce the typical asthma attack. These varied responses of the lung to antigens are related to the multiple forms in which antibodies are made in the body. Four important classes of proteins are now recognized as antibodies which perform different functions. The antibodies are called immunoglobulins (Ig), and the four classes are IgM, IgG, IgA, and IgE. Each kind of immunoglobulin is made by a different body cell.

In work previously performed in our laboratories mice exposed to 0.4 and 0.64 ppm of ozone did, indeed, experience immunological alterations as shown by rising amounts of the IgA class of antibodies in respiratory secretions, increasing number of IgA producing cells in the lungs, and accumulations of new lymphoid tissue along the airways. So far as we are aware, this is the first definitive evidence to show that changes resulting from an oxidant air pollutant are associated with increased immunological activity in the lung. Reprint requests indicate that this study has received world-wide attention (Archives of Environmental Health, reprint attached). The mouse was chosen as the experimental animal in these studies for several technical reasons. Immunological mechanisms have been more thoroughly studied in mice than in any other species, and much that is known about immune responses in man has come from knowledge obtained as a result of mouse studies.

In the case of recovery from influenza, the IgA and IgG antibodies are of great importance in neutralizing the virus, which leads to its inactivation and removal from the lung. The IgA antibodies are referred to as "secretory antibodies" because they appear abundantly in secretions, like the respiratory secretions. The IgG class of antibodies are the most numerous class in the body and, because of their relatively smaller size, they gain access to tissues and secretions whereas the larger IgM molecules tend to stay in the vascular bed.

In the instance of asthma, the IgE antibodies are specifically responsible for the disease. Cells producing IgE are distributed along the respiratory mucous membrane in a pattern similar to the IgA producing cells. Since our studies showed that ozone damage was associated with stimulation of IgA antibodies in the respiratory tract, it became reasonable to suppose that IgE production would likewise be enhanced and thus increase the numbers of sufferers from allergic lung disease.

Original Proposal:

The original proposal for the studies reported here was to use mice as a model animal and:

a. Investigate the effects of ozone and sulfuric acid aerosol in enhancing the syndrome of extrinsic asthma.

b. Examine the effects of ozone and sulfuric acid aerosol on altering the severity and pathogenesis of influenza.

Major Findings:

Major findings of these studies are:

1. Ozone exposure increases the incidence of allergic lung sensitization to an inhaled allergen.

a. Significantly greater numbers of animals were allergic in experimental groups where ozone exposure ranged from a high of 0.64 ppm to a low of 0.16 ppm.

b. The numbers of cells containing IgE in lung tissue (cells synthesizing antibodies responsible for the allergy) increased more in O_3 exposed animals than in animals that simply inhaled the allergen.

2. Sulfuric acid aerosol (concentrations of 0.5 and 1.0 mg/m^3) did not have a significant effect for the enhancement of allergy to an inhaled allergen.

3. Fatal influenza infections were less frequent in ozone exposed animals (0.4 and 0.64 ppm) than in mice breathing ambient air. Two factors appeared to interplay in producing this interesting effect.

a. Abnormal respiratory membranes developed as a result of 15 days of continuous ozone exposure, and this appeared to change the role of lining epithelium as host cells for influenza virus.

b. Ozone, at the concentrations used, was found to inactivate influenza virus. This effect could diminish the spread of virus within the airways.

The implications of these findings are considered in the body of the report.

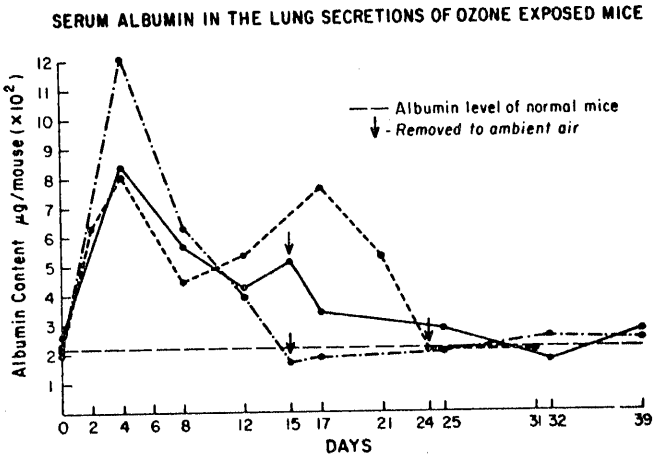
I. EFFECTS OF OZONE AND SULFURIC ACID AEROSOL ON THE SYNDROME OF EXTRINSIC ASTHMA

A. Enhancement of allergic lung sensitization by the inhalation of ozone.

Five extensive experiments were performed to analyze the enhancement of allergic lung sensitization by inhaled ozone. A detailed description of materials and methods is presented in Appendix I-A, therefore, the methods used will be presented very briefly in this section. It may be mentioned here, however, that the ozone concentrations of 0.4 and 0.64 ppm (Experiments A, B, and C) were continuously monitored on an ozone meter (Mast Development Co.). The ultraviolet photometric ozone analyzer (Dasibi Environmental Corp.) was used in Experiments D and E. Mice were allergically sensitized to an allergen either by injection (positive control groups) or by inhalation of an aerosolized allergen (lung sensitization). The allergen was ovalbumin (OA) which is a purified crystalline form of albumin obtained from the chicken egg. Ovalbumin was used to mimic the inhalation of environmental allergens such as plant pollen. A variety of proteins from plant, animal or microbial sources can be allergens in man. Ovalbumin served well as an experimental allergen since its size of 44,000 daltons placed it in that range of proteins that are small enough to be absorbed through mucous membranes and large enough to be sufficiently complex to function as immunogens(6). The contact between environmental allergens and susceptible individuals can be prolonged and nearly continuous (i.e., the pollen of plants, or the presence of animal dander from pets). However, allergen contact was necessarily limited in the experiments, and the times of contact were estimated to be periods when the antigenic stimulation would be most effective.

The circumstances for lung sensitization were unique in respect to the effects of ozone exposure. Our previous work had shown that damage to respiratory membranes could be monitored by quantitating the serum albumin levels in respiratory secretions(7). On day 4 of ozone exposure a peak of serum albumin in secretions indicated that this was a period of impaired membrane integrity, and a time when extrinsic antigen might easily gain access to immunocompetent cells in the mesenchymal tissues underlying the epithelium. The rationale for presenting airborne antigen after 3-4 days of continuous ozone exposure was based on the anticipation that sensitization might most readily occur at that time (Figure 1).

Figure 1



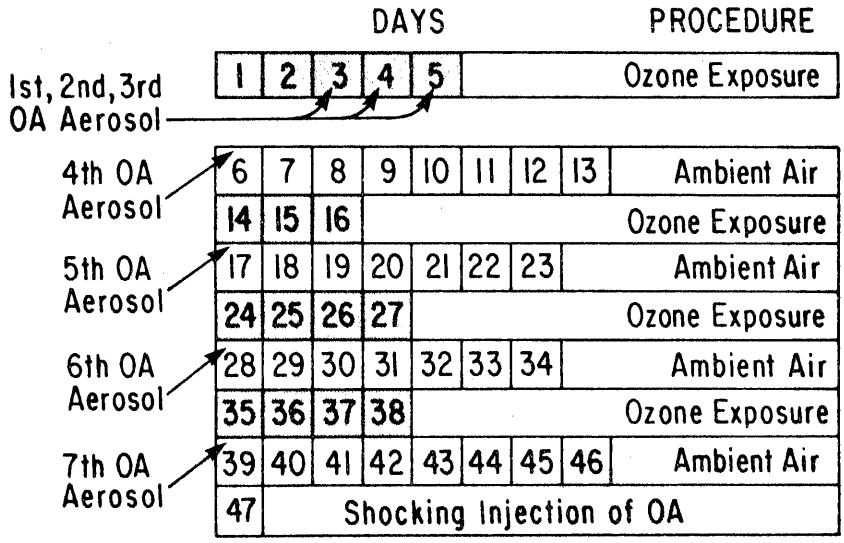
The animals were maintained in ambient air for several days before the cycle of ozone and aerosolized antigen was repeated. It has been shown that repeated morphologic damage occurs in rat lungs from 0.64 ppm of ozone when intervals in ambient air precede each ozone exposure[8]. When mice were maintained in an elevated ozone environment a peak of serum protein loss into respiratory secretions occurred on day 4, which then receded for extended periods with only minor rises above normal levels.

The OA allergen was presented as an aerosol to the mice at the time that membrane integrity was reduced during the initial exposure to ozone. Similar scheduling was used in the repeated antigen contacts. Times when the animals were held in ambient air and then returned to the ozone chambers were to simulate the intermittent phasing of low and then high air pollution episodes that may occur in the environment. This cyclic pattern of air pollution exposure and allergen presentation is exemplified in the schedule for Experiment B (Figure 2). The animals inhaled aerosolized ovalbumin (droplets of 0.5 - 3μm) for approximately 30 minutes at each aerosolization. In the 5 experiments the typical ozone exposure was continuous for 4 days during each cycle of contact with the pollutant. On a few occasions, the ozone exposure was continuous for 3 or 5 days.

Figure 2

EXPERIMENT B

Schedule of Environmental Conditions for Ozone Exposure and Aerosol Sensitization to Ovalbumin



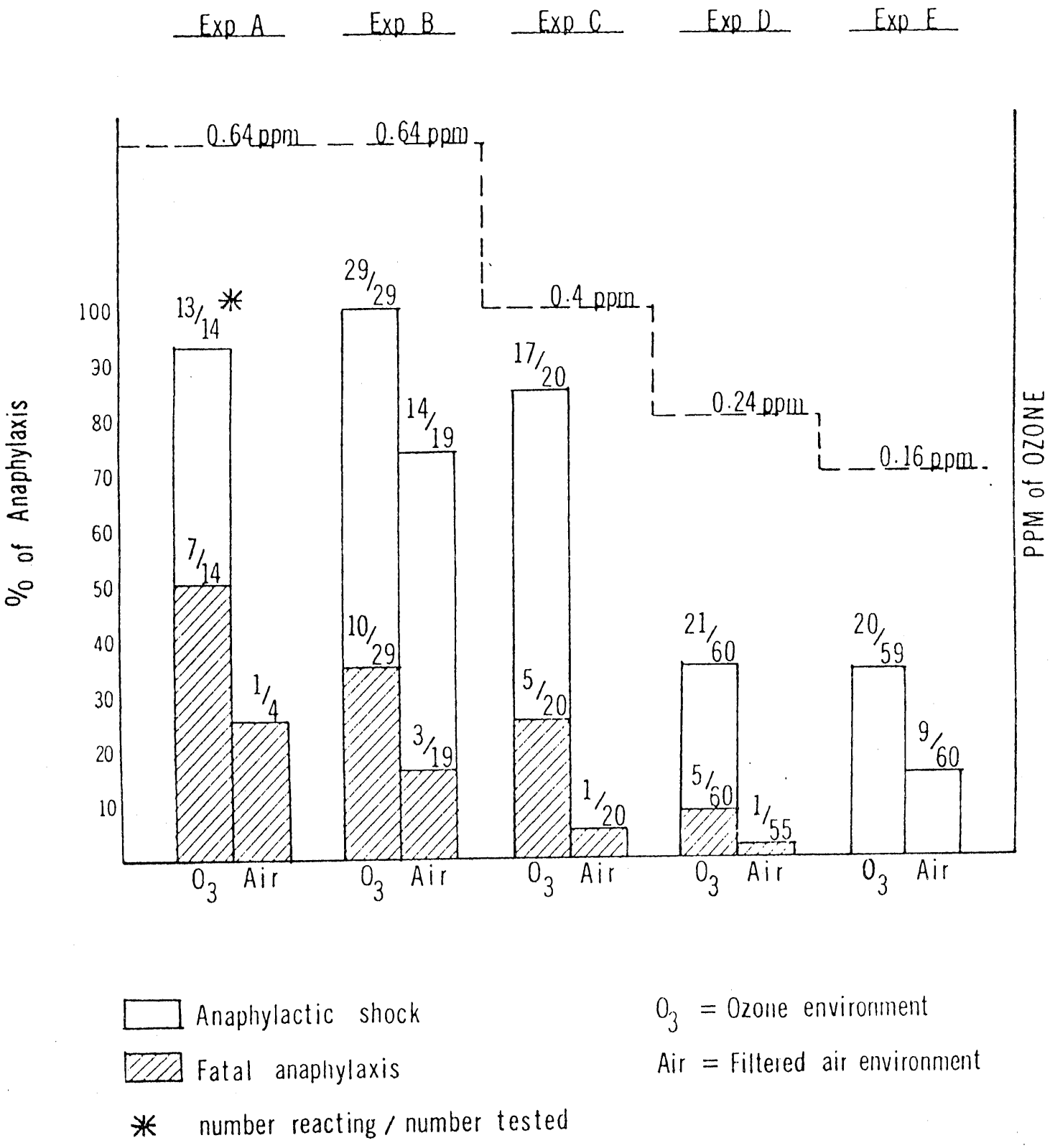
A brief account of events in this type of allergy includes the following considerations. The term "reaginic antibodies" has been used for many years when referring to the unique antibodies known now as the IgE class of immunoglobulins. IgE is well established as the homocytotropic antibody responsible for sensitizing individuals for asthmatic attacks and anaphylactic shock[9]. The B-lymphocytes synthesizing IgE are located in bronchus-associated lymphoid tissue of the lung under the epithelial

lining of the airways[10]. Their distribution in the body is similar to that of IgA producing cells. Antigenic stimulation of these cells follows the inhalation of allergens which gain access to the subepithelial spaces and contact responsive T and B lymphocytes. This leads to the synthesis of IgE with specificity for the allergen. The IgE molecules fix to mast cells by receptors on the Fc portion of the antibody molecules. Such a mast cell is sensitized. If the allergen is reintroduced, as by inhalation, the resulting antigen-antibody reaction causes the mast cell to degranulate and release pharmacologically active substances that produce the tissue responses seen in an asthma attack. An individual experiencing asthma for inhalation of the allergen can also experience anaphylactic shock if the allergen is injected in sufficient quantity. Thus, shock is associated with generalized anaphylaxis, and asthma is a syndrome of local anaphylaxis.

Since the lung is not a dominant shock organ in allergically sensitized mice, it was necessary to reveal allergic sensitization by another means. The test procedure chosen was induction of anaphylactic shock following the intravenous injection of OA. In sensitized animals the signs of increased respiration were apparent 1 to 2 min after the injection of antigen. Cyanosis was obvious as darkening of the eye and ear color, and the mice became increasingly listless and then prostrate. Some of the shocked mice positioned themselves with the head propped up in a corner of the cage. This was thought to be an effort to relieve anoxia. Within 10 min most of the severely sensitized animals were prostrate. Deaths occurred 20 to 40 min after the injections. The mice were watched carefully over a 2-hr period, and those destined to survive the anaphylactic shock gradually increased their activity as the syndrome subsided. A few survivors gained a normal appearance within 40 min, but there were also severely affected individuals that remained cyanotic and immobile, with ruffled fur, for more than 2 hours.

Each experiment required that a minimum of four animal groups be tested for anaphylactic sensitivity. The positive-control group had been sensitized by two injections of ovalbumin, and the development of shock in nearly all members of this group demonstrated that a functional allergic disease state had been established. Normal mice were injected as the negative-control group, and their failure to display any ill effects from the ovalbumin injection showed that the injected material was free from inherent toxicity. Important groups were those that had simply been exposed to aerosolized antigen, since some members of those test populations would, as expected, become allergically sensitized. Comparison of these filtered ambient air control groups with animals that had additionally been exposed to ozone were the critical test for revealing enhancement of the allergic state by ozone. The initial experiments were run at 0.64 ppm of ozone (Experiments A and B). When a significant level of enhancement was obtained in Experiment A, and confirmed in Experiment B, it became reasonable to move toward the detection of the threshold level for an ozone effect, as shown in the graph (Figure 3), subsequent studies were made at ozone concentrations of 0.4, 0.24, and 0.16 ppm. As the ozone concentration was diminished it was anticipated that smaller percentages of the test population would respond and, therefore, it was prudent to increase the numbers of test animals in order that valid statistical analyses could be made. The numbers of animals used in each experiment are presented in Figure 3. As shown in Table 1, all 5 of these experiments showed a significant level of allergic

Figure 3
Anaphylactic Reactivity of Mice Following Allergen Contact and Exposure to Ozone



enhancement (probability calculated by the exact method at the Statistical Laboratory, Division of Statistics, UC Davis). See appendix I-A for an example experimental protocol.

It should be stated that portions of this work, and implications of the study, aroused considerable interest when presented at the International Immunology Congress in Paris (July, 1980). The results of Experiments A, B, and C have been published in the Journal of Environmental Pathology and Toxicology (reprint attached).

Two additional points of information were obtained regarding the development of the allergic state. The frequency with which children

TABLE 1

Statistics on Allergic Sensitization of Mice Maintained in Filtered Ambient Air Versus Mice Exposed to Ozone[1]

	Number of Mice				
Experiment	Ozone Exposed Groups	Filtered Air Groups	Ozone Level	Probability[2]	Significance
A	14	4	0.64 ppm	P<0.018	+
B	29	19	0.64 ppm	P<0.007	+
C	20	20	0.4 ppm	P<0.001	+
D	60	55	0.24 ppm	P<0.0001	+
E	59	60	0.16 ppm	P<0.0195	+

[1]In a given experiment the contact with aerosolized allergen was equal for the two compared groups. Ozone exposures were continuous for 3-4 day periods, which were repeated intermittently in 4 cycles over approximately 6 weeks.

[2]Probability calculated by the exact method. Comparison groups are ozone exposed mice versus filtered air control mice.

display IgE mediated allergies raised the question of an age factor, which might also pertain to the mice. In Experiment D half of the mice were young (5 weeks at the start of the experiment) while the other half were adults (12 weeks). An age factor was not apparent since there was no significant difference in the responses of the different age groups.

Another point of information concerned allergic enhancement at 0.16 ppm of ozone. Enhancement was confirmed in groups of mice that had received injections of an adjuvant to boost the IgE responsiveness in the animals. Inactivated Bordetella pertussis cells have been shown by others to bring about this adjuvant effect and, therefore, to exaggerate the allergic responsiveness. (It is of interest that our source of Bordetella cells was

the commerically available whooping cough vaccine that is used to immunize children.) This adjuvant did boost the responsiveness of the mice; but once again, the reactivity was significantly greater in the ozone exposed mice (probability of <0.005 when compared to the ambient air controls) which once again revealed the effect of ozone enhancement.

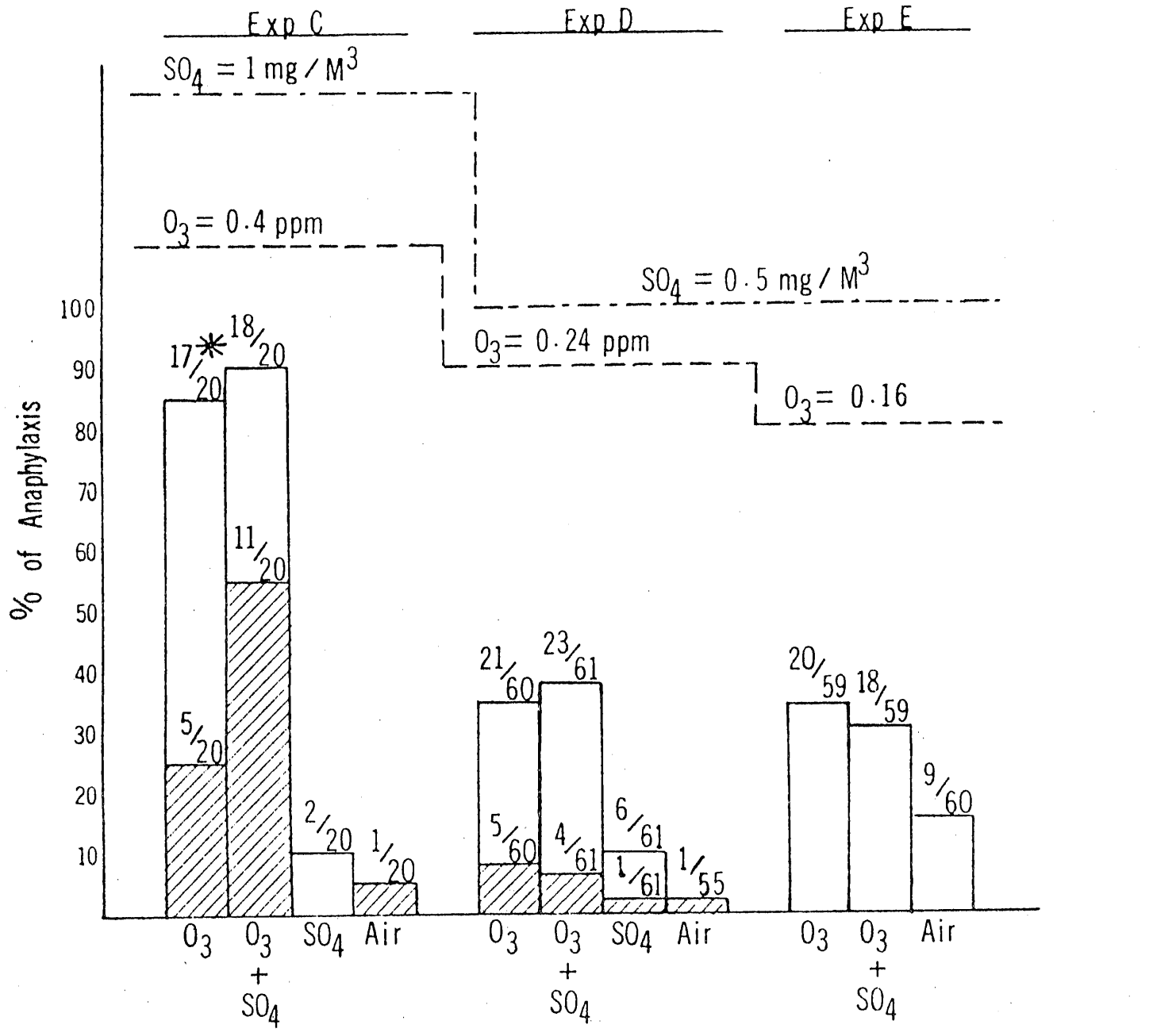
I. B. Studies on the Effects of Sulfuric Acid Aerosols on Allergic Lung Sensitization.

Sulfuric acid aerosol studies were included in the protocols for Experients C, D, and E, which were previously described for the ozone investigations. Groups of mice were exposed to sulfuric acid aerosols of 0.5 mg and 1 mg/m^3, either as the only air pollutant or in combination with ozone. It was necessary to use specially designed chambers where the concentration of sulfuric acid was monitored on cascade impactors or membrane filters[11]. The sulfuric acid aerosol droplets had a count median diameter (CMD) of 0.041 + 0.005 μm with a geometric standard deviation of 1.86 + 0.06. In Experiment C ozone was monitored by the Mast Ozone Meter, and in Experiments D and E the Dasibi Ultraviolet Photometric Ozone Analyzer was used. Aerosolized control mice were housed in identical chambers containing ambient air. These mice were exposed to aerosol sensitizing antigen, along with animals exposed to the air pollutants. As in the ozone studies, the mice were exposed to the sulfuric acid aerosol continuously for 4 days, and then aerosolized ovalbumin as the allergen was presented by inhalation for 30 minutes. This cycle was repeated 4 times over a period of approximately 6 weeks. The animals were breathing filtered ambient air during the periods between exposure to the air pollutants. Anaphylactic sensitivity was tested by injecting ovalbumin. The conditions for each experiment are presented on Figure 4 and Table 2.

The group receiving the combined pollutants in Experiment C was highly sensitized (90%), but the group exposed to ozone as a single pollutant was also very reactive (85%). The shock pattern was more severe in the combined group with 55% dying as compared to 25% for the group receiving ozone alone. When subjected to Chi-square analysis, this difference in mortality did not prove to be significant at the 5% level (X^2=3.75, probability between 5% and 10%). Since this difference in allergic severity approached significance, it was deemed necessary to pursue the question further. A synergistic effect would necessarily have to manifest itself at pollutant levels more nearly approximating those encountered in the environment to warrant concern in evaluating health effects. Consequently, the sulfate level was reduced to 0.5 mg/m^3. If a synergistic effect by the two pollutants were to occur, it was thought that this might be more apparent if the ozone levels were lower. In Experiments D and E the ozone exposures were reduced to 0.24 and 0.16 ppm, respectively. As shown in Figure 4 and Table 2, the suggestion of a synergistic effect was not revealed when the pollutant levels were reduced to levels closer to those of the environment. From Comparison Groups I it is apparent that sulfuric acid exposure alone did not significantly increase allergic sensitization above the responses of animals maintained in filtered air. The Group II comparisons did show that allergic enhancement occurred in an ozone plus sulfuric acid aerosol environment. As one would expect, the Group III comparisons showed that the combined pollutants produce responses significantly greater than those of

Figure 4

Anaphylactic Reactivity of Mice Following Allergen Contact and Exposure to Sulfuric Acid Aerosol



the animals encountering sulfuric acid aerosol alone. However, when the ozone groups were compared to the combined pollutant groups (Comparison Groups IV) it was seen that the significant differences disappeared. In addition, significant differences in the severity of the shock (mortality) in the Group IV sets could not be demonstrated.

It was concluded that the allergic enhancement seen in the combined ozone and sulfate groups could be explained on the basis of the ozone effect, and that the sulfate alone did not appear to alter the lungs in a manner that would lead to augmented IgE responsiveness. This negative finding with respect to sulfuric acid aerosol further delineated the uniqueness of effects on lung tissue from ozone inhalation. This information will be submitted for publication.

TABLE 2

Statistics on Allergic Sensitization of Mice Exposed to Sulfuric Acid Aerosol[1]

Experiment	Pollutant Levels	Comparison Groups[2]	Chi-Square Values	Significance[3]
Exp. C	H_2SO_4= 1 mg/m^3 Ozone = 0.4 ppm	I. Air Cont. (N=20) versus SO_4 (N=20)	X^2=0.385	-
		II. Air Cont. (N=20) versus O_3 + SO_4 (N=20)	X^2=28.9	+
		III. SO_4 (N=20) versus O_3 + SO_4 (N=20)	X^2=25.6	+
		IV. O_3 (N=20) versus O_3 + SO_4 (N=20)	X^2=0.229	-
Exp. D	H_2SO_4= 0.5 mg/m^3 Ozone = 0.24 ppm	I. Air Cont. (N=55) versus SO_4 (N=61)	X^2=3.27	-
		II. Air Cont. (N=55) versus O_3 + SO_4 (N=61)	X^2=13.1	+
		III. SO_4 (N=61) versus O_3 + SO_4 (N=61)	X^2=22.7	+
		IV. O_3 (N=60) versus O_3 + SO_4 (N=61)	X^2=0.10	-
Exp. E	H_2SO_4= 0.5 mg/m^3 Ozone = 0.16 ppm	II. Air Cont. (N=60) versus O_3 + SO_4 (N=59)	X^2=4.08	+
		IV. O_3 (N=59) versus O_3 + SO_4 (N=59)	X^2=0.16	-

[1] In a given experiment the contact with aerosolized allergen was equal for the compared groups and consisted of 4 contact periods with a duration of 30 minutes each. Sulfuric acid aerosols and ozone exposures were continuous for 4 day periods, which were repeated intermittently in 4 cycles over approximately 6 weeks.

[2] SO_4=Sulfuric acid aerosol environment
O_3 + SO_4=Combined ozone and sulfuric acid aerosol environment
O_3=Ozone environment
Air Cont.=Filtered air control environment

[3] - equals not significant at 5% level
+ equals significant at 5% level or less

I. C. Immunoglobulin E-containing cells in mouse lungs following allergen inhalation and ozone exposure.

After demonstrating that ozone damage to the lungs could lead to enhanced allergic reactivity toward an inhaled antigen, it became of great interest to correlate this with evidence that IgE synthesis had actually been stimulated in the lung tissue. To do this we decided to reveal the cells in the lungs containing IgE in their cytoplasm by means of the fluorescent antibody technique. The procedure required the use of antibodies directed against a restricted part of the IgE molecule (the heavy chain, known as the epsilon chain) to which fluorescein (a fluorescing dye) could be bound by a conjugation process. Since there was no commercial source for the anti-IgE reagent, it was necessary for us to prepare the antiserum in goats, which were immunized with mouse IgE that we obtained from mice immunized with ovalbumin. The steps in this procedure are described in Appendix B. Various aspects of this procedure were unique, and they have been brought together in a manuscript entitled "Immunogenic collagen in induced ascitic fluid: Concurrent with preparation of anti-murine IgE" which is "In Press" with the American Journal of Veterinary Research (manuscript attached).

Lungs were analyzed from animals in Experiments A, B, and C. It was desirable to study the lungs from animals that were reared free of lung infections since lung disease would give rise to immune responses. The specific pathogen free (SPF) mice used here were monitored by the suppliers to maintain their freedom from a group of respiratory pathogens.

Following the last ozone exposure in Experiments A, B, and C, aliquots of mice from each test group were removed for tissue examination. The remaining mice received a final aerosolization of OA and were maintained in ambient air for 6-8 days. They were then injected IV with 2 mg of OA in 0.1 ml to detect systemic anaphylaxis.

IgE-Containing Cells in Normal Lungs. Few IgE-containing cells were encountered in the lungs of normal mice. Among the sections examined from 12 mice, there were 3 that contained no fluorescing cells. The average count was 6.2 cells/lung section. The IgE-containing cells among these unmanipulated control mice tended to be located in the adventitium of the airways. Eighty-four percent of the cells were airway-related and the remainder were scattered in the alveolar walls of the lung parenchyma.

IgE-Containing Cells in the Lungs of Mice that had Inhaled Aerosolized OA. The numbers of IgE-containing cells increased among mice that had encountered aerosolized OA on four to seven occasions. The mean count was 47 cells per section among 21 examined lungs. The distribution of cells was modified from those seen in normal lungs with 43% related to the airways.

IgE-Containing Cells in the Lungs of Mice that Had Been Exposed to Ozone and Had Inhaled Aerosolized OA. A marked increase in IgE responsiveness was apparent in lungs affected by ozone exposure in addition to the antigenic stimulus of aerosolized OA. The mean cell count was 156 cells per lung section among 23 lungs studied. Careful examination revealed

increases in numbers of mononuclear cells in mesenchymal regions of the lung including spaces adjacent to airways, in proximity to small blood vessels, and in alveolar walls. Sixty percent of the cells were airway-related. However, lung sites distal to the conducting airways were involved in IgE synthesis. Fluroescing cells were observed in nests of mononuclear cells clustering outside of blood vessels, within alveolar walls, and sometimes within blood vessels.

IgE-Containing Cells in the Lungs of Mice Injected Intraperitoneally with ovalbumin. Positive control mice were injected twice IP (3-week interval) with 1.0 μg OA in 1 mg of alum precipitate. Counts of IgE-containing cells in the lungs of 18 animals showed a mean value of 15 cells per section, which were primarily airway-related. These animals were highly sensitized as revealed by IV injections of OA into other members of the immunized groups. The injected antigen was, no doubt, systemically distributed to involve sites such as mediastinal lymph nodes, spleen, and other lymphoid tissues in the process of IgE synthesis. The number of IgE-containing cells in the lungs was higher than those encountered in normal mice (6.2 cells per section), but IgE synthesis in lungs was probably a small part of the IgE responsiveness of these mice.

Statistical Summary of IgE-Containing Cells in Lungs. The counts of IgE-containing cells have been presented as the number per section. A more precise value was obtained by calculating the number of cells per square millimeter of lung tissue. It was also of interest to compare the numbers of airway-associated cells in the various treatment groups by calculating the number per millimeter of airway. These determinations were necessary because there was considerable variation in the area and airway perimeter among the lung sections.

In each experiment the numbers of IgE-containing cells per square millimeter of lung increased from normal to OA-aerosolized to ozone-exposed and OA-aerosolized lung. The conditions of each experiment differed and this was reflected in variability in the magnitude of increase in the responding cells (Table 3). Nevertheless, the trend of the data remained the same regardless of the variation in experimental design. A composite view of the cell dynamics was obtained by combining the groups from each experiment (Table 4).

TABLE 3

Increasing Ratios of IgE Containing Cells in the Lungs of Mice Following Inhalation of Allergen and Exposure to Ozone

Comparison Groups	Experiment		
	A O_3=0.64 ppm	B O_3=0.64 ppm	C O_3=0.4 ppm
Normal to Aerosolized OA[1]	1.2	39.0	6.2
Aerosolized OA to Ozone + Aerosolized OA[2]	3.0	1.6	100.0
Normal to Ozone + Aerosolized OA[3]	3.5	64.8	623.0

[1]Cells counted per mm^2 in normal mouse lung divided into cells counted per mm^2 in lungs of mice aerosolized with ovalbumin allergen (OA).

[2]Cells counted per mm^2 in mouse lung from animals aerosolized with OA divided into cell counts per mm^2 in lungs of mice that had been exposed to ozone and had inhaled aersolized OA.

[3]Cells counted per mm^2 in normal mouse lung divided into cells counted per mm^2 in lungs of mice that had been exposed to ozone and had inhaled aerosolized OA.

Summary on the IgE-Containing Cells in Lung Tissue. Histological observations revealed that the mouse lungs contained more mononuclear cells as the number of IgE-containing cells increased. The morphological appearance of cells revealed by immunofluorescence with an anti-IgE reagent was compatible with lymphocytes and plasma cells. Among three experiments, where some variations in design existed, there was a consistent general pattern of response. Composite data from the studies showed that a 9.4-fold increase of IgE-containing cells developed in mice examined 25-37 days following the first contact with aerosolized OA. When mice were additionally exposed to 0.4 ppm or 0.64 ppm of ozone, there was a 34.2-fold increase in IgE-containing cells (Table 4). The design modifications showed that the cycle of events leading to enhancement of immediate hypersensitivity persisted through such changes. Exposure of mice to ozone levels of 0.4 or 0.64 ppm enhanced immune responsiveness with IgE formation. This was apparent from the positive correlation between ozone exposure and the numbers of IgE-containing cells in the lungs. Much of the IgE synthesis was stimulated along the airways. A 7.3-fold increase of airway-related IgE cells followed the ozone exposure (Table 4). In addition, a significant increase in anaphylactic sensitivity was detected in the three experiments when antigen-stimulated and ozone-exposed animals were compared to mice that had received antigen only.

TABLE 4

Dynamics of IgE Containing Cells in Mouse Lungs

Mouse Groups	IgE cells/mm of tissue		Airway related IgE cells/mm of airway	
	Mean[1]		Mean[1]	
Normal	0.196 (± 0.186)[2]		0.299 (± 0.369)[2]	
Aerosolized OA	1.85 (± 4.13)		0.460 (± 0.723)	
Ozone + Aerosolized OA	6.71 (± 9.79)		3.36 (± 3.77)	
	probability[3]	fold increase	probability[3]	fold increase
Normal vs Aerosolized OA	0.038	9.4	0.202	1.5
Aerosolized OA vs Ozone + Aerosolized OA	0.018	3.6	0.0004	7.3
Normal vs Ozone + Aerosolized OA	0.010	34.2	0.001	11.2

[1]Mean values for cumulative data in Experiments A, B, and C

[2]Standard deviation

[3]Student T test

This study is "In Press" and will appear soon in the International Archives of Allergy and Applied Immunology.

I. D. Cell enumeration by immunofluorescence

During the period of these experimental studies the use of hybridomas has come into use in experimental immunology. In this procedure an antibody producing cell is fused with a neoplastic cell (a cancer cell) to make a hybrid. The hybrid can be cloned and maintained perpetually in tissue culture because the cancer cell does not die out. In this hybridized state the antibody formed maintains the strict antibody specificity of the originally cloned lymphocyte. A cloned antibody product is now prepared commercially (Miles Laboratories) as an anti-mouse IgE reagent. Such a reagent will become the standard by which different laboratories will

compare results and it behooves us to change from the anti-IgE serum produced in this laboratory to the new hybridoma produced antibody.

We have already purchased the new anti-IgE and have conjugated it to fluorescein for use as a fluorescent antibody. On comparative studies, we seem to be delineating the same cells with our own anti-IgE and the hybridoma anti-IgE. Consequently, we feel that this work can continue with the new reagent and that it will reflect an extension of our previous studies.

However, we are experiencing a difficulty regarding the status of the experimental animals. In Experiments A, B, and C we were able to assume that the specific pathogen free mice were essentially naive with respect to IgE responses in the lungs because the examination of the unmanipulated controls had very few IgE-containing cells. Furthermore, the increases in IgE cells correlated with experimental events. However, the mice in Experiments D and E have been found to contain considerable numbers of IgE-cells in the lungs as they arrive from the supplier. The test that we have used for all enumeration is specific for the IgE class of antibodies and we assume, by inference, that rising numbers relates to IgE synthesis against our test antigen, ovalbumin. Newly arrived animals, showing IgE cells in their lungs, have been stimulated by some unidentified antigen. It is most likely that these animals are responding to some parasite infections that have become established in the animal colony. We found whipworms and mite infections in the animals as they were purchased. Telephone conversations with the supplier in Pennslyvania made it clear that they were likewise aware that extraneous infections had gotten into their specific pathogen free colony. The company was erecting new buildings and starting over again with new animal stock. We recently examined a few animals from this fresh colony and find that they still contain numerous IgE-containing cells. This creates problems in establishing baseline levels.

The obvious way to proceed around such a dilemma is to render our test specific for IgE containing cells that are making antibodies against ovalbumin. To explore the possibility of this approach we placed a fluorescent label (rhodamine) on ovalbumin itself. The idea was to look for double-labeled cells which would appear apple-green from the fluorescein to show that they were synthesizing IgE; and would also appear red from the rhodamine, to show that they were making antibodies to ovalbumin. Regrettably, the procedure will not be that simple because we find that ovalbumin non-specifically adheres to several cells in the lung tissues of normal mice. This non-specific adsorption of ovalbumin appears to result from a charge effect on the highly electro-negative surface of the ovalbumin molecule (isoelectric point = pI 4.5). It is thought that this work needs to be pursued further. In our proposal for continuation of these studies we are suggesting a method for reducing part of the negative charge on ovalbumin. The purpose is to eliminate this problem of non-immunological binding of ovalbumin to the cells of non-immunized animals. Until this obstacle to cell enumeration is corrected, the counts from the lungs in Experiments D & E must be delayed.

I. E. Preliminary study into the mechanism of increased allergic sensitization from ozone exposure.

Work from other laboratories has indicated that the allergies mediated by the IgE class of antibodies are under strong immuno-regulation by suppressor and helper T-cells (thymus derived lymphocytes). Stated briefly, the non-allergic individual is presumed to be in a balance wherein strong suppressor cell function dampens the IgE responses and inhibits the development of overt IgE synthesis to environmental antigens. On the other hand, the allergic person has diminished suppressor cell activity (heritable?) so that helper T-cells can function toward raising IgE synthesis, which leads to the sensitization of mast cells with IgE antibodies, and thus to an allergic disease process. The SJL strain of mice has been reported to be a "low responder" genetic line with respect to IgE synthesis. This is described as being due to a highly efficient suppressor T cell system which keeps the animals in a non-allergic state analogous to the non-allergic human subject. It has also been reported that the suppressor T cells are a radiosensitive population. They can be eliminated by sublethal X-irradiation, which then converts the SJL mouse to a "high responder" state for IgE synthesis.

Use of the SJL mouse as a model to study ozone effects was begun on the premise that suppressor T-cells in the lungs might be highly susceptible to ozone damage and thus affect the regulation of local IgE synthesis in the lung by permitting greater responses.

At the time of this writing we are still examining the results of an initial experiment which has led to indefinitive results. The basic problem seems to lie with the mice, which did not perform as reported by other laboratories. Rather than functioning as low responders, these animals made a moderate to strong IgE response. Furthermore, X-irradiation did not lead to enhancement of their IgE synthesis as others had reported. More work will be required to unravel the difficulties here, since the use of this model does bear on important questions of IgE regulation which may affect human lung disease.

II. EFFECTS ON INFLUENZA VIRUS INDUCED PNEUMONIA FROM THE INHALATION OF OZONE

Epidemiological data have indicated in many cases that the rate and severity of respiratory viral infections, such as influenza, increase appreciably when high levels of air pollutants are present. Mice are particularly suitable as mammalian hosts for experimental studies with the virus causing human influenza[12]. It is of special interest that the influenza virus affects many of the same cells in the respiratory system that are damaged by ozone inhalation. We have investigated the pathogenesis of influenza virus infection in mice by exposing them to aerosols of influenza virus, and examining the infected respiratory tissues.

II. A. Studies on influenza pneumonitis.

These studies had to be made prior to evaluating ozone effects since the parameters to be investigated had to be followed first in the uncomplicated disease process to understand the normal host responses to the infection. During this project period the following papers have either been published or are "in press". Details of the materials and methods used for these investigations are presented in Appendix II-A.

1. Yilma, T., Y. C. Zee, and J. W. Osebold. 1979. Immunofluorescence determination of the pathogenesis of infection with influenza virus in mice following exposure to aerosolized virus. J. Infect. Dis. 139: 458-464.

By enumeration of the number of airways, alveolar macrophages, and alveolar cells containing influenza viral antigens at different intervals after exposure to the viral aerosol, it was determined that viral replication occurred initially in the epithelial cells lining the airways and later extended to the alveolar macrophages and alveolar cells. This survey of the dynamics of influenza viral infection by aerosol indicated that the viral infection in mice was a descending process.

2. Owen, S. L., J. W. Osebold, and Y. C Zee. 1981. Dynamics of B-Lymphocytes in the lungs of mice exposed to aerosolized influenza virus. Infect. and Immun. "In Press".

Immunoglobulin-containing cells were revealed by immunofluorescence in lung sections from mice infected with influenza virus by the aerosol route. The numbers of IgA and IgM containing cells were increasing by day 3 of the infection, while IgG containing cells appeared a few days later. The responding B-cell populations appeared in two principal locations: along major airways, and in consolidated lesions within lung parenchyma. Immunoglobulin A containing cells were the most numerous isotype, occurring predominantly in the lamina propria of the airways. Immunoglobulin G containing cells were the least frequently encountered class along airways, and appeared most often within consolidated lung lesions in clustered groupings. Cells staining for IgM appeared along the airways and in lung lesions. The population of IgM containing cells declined approximately 30 days following infection. Cells producing IgA and IgG were still numerous on day 46. Assays for virus reactive antibodies in lung secretions were positive on day 8 of the infection. The IgM titers were the first to decline, but virus binding antibodies for all classes were still present on day 33.

Aerosolized influenza virus is widely distributed in the parenchyma of the lung and along the airways. As the virus enters susceptible cells and proliferates, there is an expansion in the amount of antigen that is locally available. Furthermore, systemic antigenic stimulation follows as virus spills out of the lungs to produce viremia.

Lymphocytes located in the lung are, no doubt, the first to respond when the viral antigen accumulates along the airways. As virus replication progresses, general recruitment of lymphocytes into the lung would follow as cellular traffic expands into inflammatory sites. The extensive parenchymal lesions would be important sites for cytotoxic T-cell activity, in addition to serving as a major new region for antibody synthesis. Infective virus had been eliminated from the tissues in surviving animals a few days following the last deaths. While all factors contributing to death in influenza pneumonitis are not known, the loss of respiratory exchange tissue from edema and consolidated lesion formation is of major importance. Delay in mounting an immune response could lead to overwhelming of the animal by massive lesion production and the associated inflammatory changes.

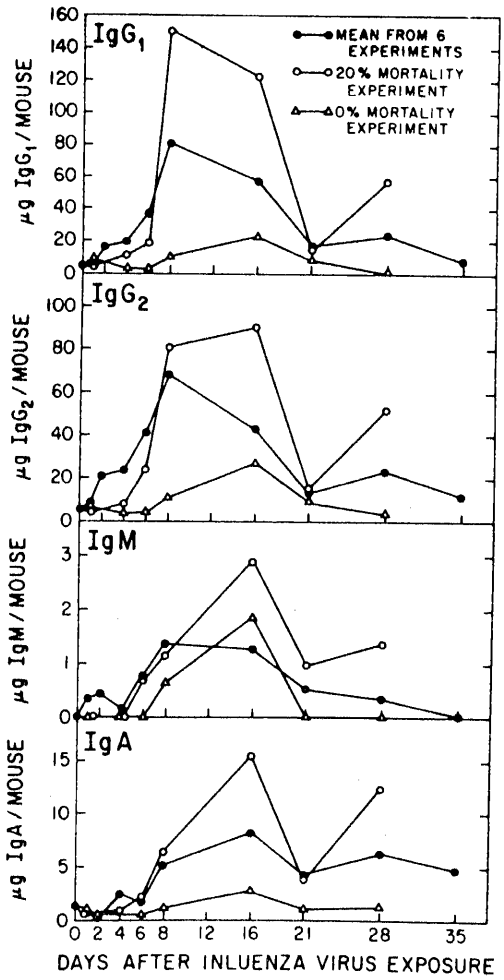
This study presents evidence that the limited number of immunologically competent cells in normal lungs is subject to great expansion in response to diffuse invasion by an infectious agent. The B-cell events were diverse with respect to class and timing. Antibody was formed in an apparent response by resident immunocytes, as well as cells recruited from the general lymphocyte traffic into sites of tissue damage and concentration of viral antigen.

Antibody synthesis of the IgG and IgA classes was seen to continue for an extended period beyond the time of pneumonic crisis, and after infective virus had disappeared. The special transport mechanism for IgA would maintain virus-specific antibodies in the secretions during convalescence, while the locally formed IgG could contribute in a major way to the serum titers, in addition to those of the respiratory secretions.

3. Zee, Y. C., J. W. Osebold, and W. M. Dotson. 1979. Antibody responses and interferon titers in the respiratory tract of mice after aerosolized exposure to influenza virus. Infect. Immun. 25: 202-207.

Immunoglobulin levels and interferon in external secretions have been shown to be important in several respiratory viral infections. We undertook this study to analyze the events after aerosol exposure of mice to the human influenza virus in six animal groups in which mortality rates ranged from 0 to 24%. Our previous studies demonstrated that low concentrations of certain immunoglobulins were present in normal mouse lung lavage fluids. The mean values for IgG_1, IgG_2, and IgA in normal mouse lung lavages were 4.09, 5.45, and 2.36 µg/mouse, respectively, IgM and interferon were not detected. These values may be compared with an analysis for protein content in normal mouse serum (milligrams per milliliter): albumin, 35.1; IgG_1, 2.53; IgG_2, 1.85; IgA, 0.65; and IgM, 0.06. After influenza virus infection, a significant elevation of each immunoglobulin class and interferon was noticed, although the extent of response was substantially influenced by the severity of the infectious process (Figure 5).

Figure 5

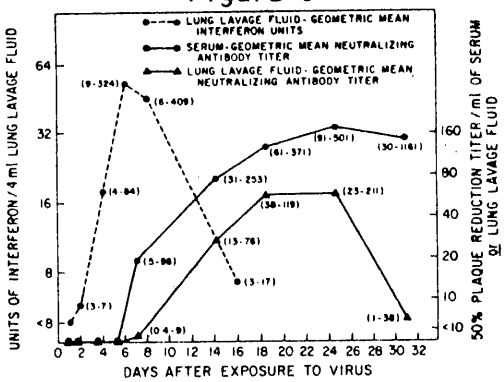


Immunoglobulin content in lung lavage fluid from influenza virus-infected mice. Normal mice were used to determine the zero-time value.

In experiments with low mortality rates, immunoglobulin levels in the lung lavage fluids were minimal. The lower virus titers in the lung tissues of these animals may have been responsible for the lack of immunological responses, since there appears to be an association between the amount of antigen present and the level of immunoglobulin response in the respiratory tract. Marked increases in IgG_1, IgG_2, IgA, and IgM levels occurred in experiments with higher mortality rates (16, 20, and 24%). Immunoglobulin concentrations became detectable on day 6 after exposure to virus and reached their peaks on day 8 or 16, depending on the class of immunoglobulins. Maximum viral titers in these experiments were observed on day 4 after exposure.

There was much IgG in the respiratory secretions during the early phases of the process, but most of this was associated with increased vascular permeability and accumulations of serum proteins as part of the pneumonitis induced by the infection. Acute respiratory inflammation with its concomitant exudation of plasma proteins into the respiratory tract has also been observed with other infectious agents. Analysis for serum albumin indicated peak levels on day 8, with high levels persisting through day 16. Fatal influenza infections clustered between days 9 and 12 postinfection. Albumin levels tended to persist somewhat above normal values during the repair processes in surviving mice.

Figure 6



Interferon in lung lavage fluid and influenza virus-neutralizing antibodies in serum and lung lavage fluid. Standard deviations are expressed as titers or units in parentheses. Data are from six experiments.

Virus-neutralizing antibodies were found on day 8 in both serum and respiratory secretions. The titer was low at the time in the secretions, but much of the antibody available then would be expected to bind to viral antigen and thus become unavailable to assay as free antibody (Figure 6).

Antibodies detected as virus neutralizing and those designated by immunoglobulin class as reactive with influenza virus (by the indirect fluorescent antibody method) were the result of antigenic stimulation from the virus. These antibodies were detected 6 to 8 days into the process and at a time shortly before the deaths, which began on day 9. Titers for these antibodies tended to peak 3 to 4 weeks after the virus exposure, which indicated continued antibody synthesis from the stimulation of viral antigen that had been eliminated from the tissues 2 weeks previously.

Interferon has been presumed to play a major role in recovery from viral respiratory infection, especially in the primary infection with a specific respiratory virus. Early events with regard to the availability of interferon and the rapid production of antibodies were probably key determinants in governing the severity of the pneumonitis in different sets of mice. Early antibody to virus was detected on day 6 in the respiratory secretions of the groups experiencing 0 and 2.5% mortality. Both systemic and local antibody production would be expected to contribute to immunoglobulins in the respiratory secretions.

The assay curves for IgM and IgA suggested that much of these immunoglobulins came from local synthesis.

II. B. Current status of studies on the effects of ozone inhalation on influenza.

The number of fatal infections in the mice rises as the lesions become more acute and extensive. From the observations on human clinical cases it could be expected that ozone damage to the lungs would increase the severity of the influenza process. However, in the experimental situation, where relatively high ozone levels were used, the opposite effect was seen to occur. That is, fatal infections were less frequent in virus infected animals that were also inhaling ozone. Table 5 presents results from four experiments in which the mortalities varied from 9% to 100% among mice breathing ambient air (See Appendix II-B for sample experimental protocol).

This surprising result is repeatable and we are now quite convinced that ozone alters the pathogenesis of influenza pneumonitis under the specific set of conditions used in these experiments. Some progress has been made toward understanding what is actually happening in the lungs, but, as we will explain, several uncertainties still exist. At this writing it is not clear how important these findings are to respiratory infections as encountered during air pollution episodes.

There are two lines of evidence to indicate that there is less virus involvement of the major airways when elevated ozone levels are present. The evidence relates to the distribution of lung lesions and the location of virus in the lungs.

TABLE 5

Experiment No.	Environment			
	Ozone[1]		Ambient Air[2]	
	Ozone concentration	% Mortality	% Mortality	Probability[3]
1	0.64 ppm	33% (N=40)	100% (N=40)	<0.001
2	0.64 ppm	0% (N=40)	9% (N=40)	<0.05
3	0.4 ppm	0% (N=74)	33% (N=74)	<0.001
4	0.4 ppm	19.5% (N=74)	56.1% (N=74)	<0.001

[1]Maintained in continuous ozone exposure for 15 days prior to infection with aerosolized virus and returned to ozone chamber for an additional 15 days.

[2]Maintained in filtered ambient air before and after infection.

[3]Chi-square analysis. Comparisons are only made within each experiment since the dose of virus varied between experiments.

i. Lesion Distribution

A system for scoring lung lesions was devised for the examination of stained lung sections, which were always cut to include the major lobar bronchus.

Scoring of Lesions. Numbers were assigned as follows:

1 = Lesion in contact and associated with the bronchus.

2 = Lesion in the central parenchyma, but showing an extension to the bronchus.

3 = Lesion in the central parenchyma.

4 = Lesion in the central parenchyma, but showing an extension to the pleural surface.

5 = Lesion extending along the pleural surface.

From such a scoring system a low score is indicative of lesions associated with the major airways, while a higher score suggests more peripheral lesions in the distal airways and alveolar tissue. The following scores were obtained in the four experiments described below (Table 6).

TABLE 6

Influenza Lesion Scores[1]

Experiment No.	Ozone + Virus	Ambient Air + Virus
1[2]	3.4 (N=28)	Not Done
2[2]	3.8 (N=32)	2.1 (N=20)
3[3]	3.2 (N=10)	2.9 (N=10)
4[3]	3.2 (N=12)	2.4 (N=12)
Average Score	3.4 (N=82)	2.5 (N=42)

[1]Virus exposures were constant within an experiment, but variable between experiments.

[2]Ozone concentration=0.64ppm

[3]Ozone concentration=0.4ppm

This result suggested that the usual influenza process with its major involvement along the airways may be altered by high ozone levels to produce more peripheral lesions.

ii. Virus Location

Sites of influenza virus replication were studied in two large experiments where mice were exposed to various combination sequences of ozone and/or filtered ambient air in conjunction with infection by aerosolized influenza virus.

In these experiments 8 week-old specific pathogen free mice of the Swiss-Webster line were divided into four different exposure protocols, each consisting of 120 mice. Group A (O_3 - V - O_3) was held in a continuous ozone environment (0.4 ppm, CARB ultraviolet photometric standard) for 14 days. The mice were then infected with aerosolized influenza virus and were placed back in the ozone environment for an additional 14 days. Group B (O_3 - V - F) started out identically to Group A, but after infection these mice were maintained in filtered air for 14 days. Group C (F - V - O_3) animals were initially held for 14 days in filtered air and were then exposed to the virus. After infection they were placed in the ozone environment for 14 days. Group D (F - V - F) mice functioned as the virus infected control group, which was held in filtered air throughout the study. It should be noted that statistical comparisons are made only between groups within a given experiment where animals received the same virus exposure. The mice were monitored for mortality, virus production, sites of viral replication, interferon and neutralizing antibody production. Mortality rates were determined on sets of 74 mice, since many animals from the original group of 120 were required for the other analyses.

Clinical symptoms of influenza infection in mice included huddling, ruffling of the fur on the back, dyspnea, emaciation, and stiffness or immobility prior to death. Because of the subjectivity in evaluating clinical symptoms of influenza, determinations of disease severity were based on percentages of mortality in the various treatment groups. Mortality results are reported in Tables 7 and 8.

TABLE 7
Cumulative Percent Mortality in Mice Exposed to Ozone and Infected with Influenza Virus[1]

Experiment No.	Group A O_3-V-O_3[2]	Group B O_3-V-F	Group C F-V-O_3	Group D F-V-F
3	0.0	9.3	2.6	33.0
4	19.5	46.2	10.3	56.1

[1]For each group N = 74 mice.

[2]Symbols denote sequence of events: O_3 = continuous ozone environment of 0.4 ppm for 14 days, V = exposure to virus, F = Continuous filtered air environment for 14 days.

TABLE 8

Statistics on Mortality in Mice Exposed to Ozone and Infected with Influenza Virus

Experiment	Ozone Level	Comparison Groups	Chi Square values	Probability
3	0.4 ppm	O_3 - V - O_3[2] vs F - V - F	27.8	<0.001
		O_3 - V - F vs F - V - F	13.8	<0.001
		F - V - O_3 vs F - V - F	24.9	<0.001
4	0.4 ppm	vs F - V - F		
		O_3 - V - F vs F - V - F	1.6	<0.2=N.S.[3]
		F - V - O_3 vs F - V - F	38.6	<0.001

[1]For each group N = 74 mice.
[2]Symbols denote sequence of events: O_3 = continuous ozone environment of 0.4 ppm for 14 days, V = exposure to virus, F = filtered air environment for 14 days.
[3]N.S. = not significant

As shown in Figure 7 virus titers did not vary markedly among the 4 tested groups when whole lung homogenates were assayed.

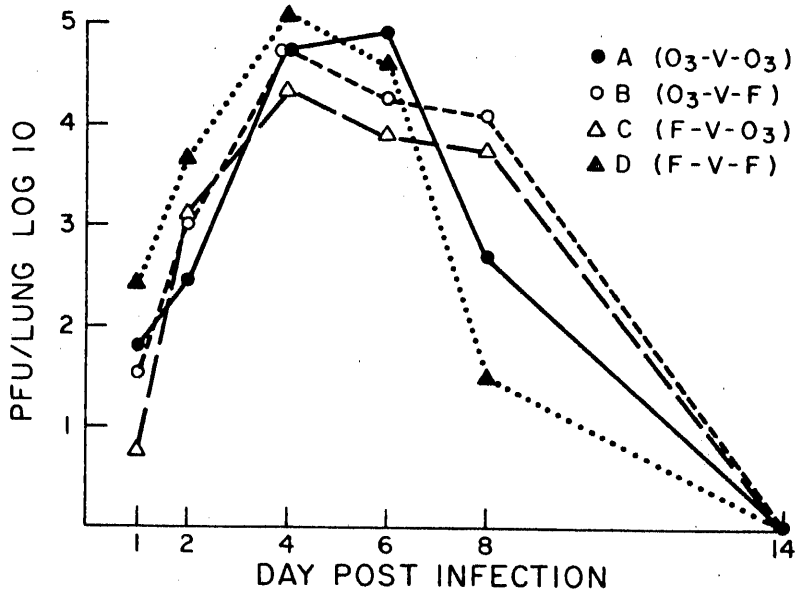


Figure 7. Titers if influenza A virus in homogenates of lungs taken from mice in Experiment #3 following exposure to aerosolized virus. The log of the virus titer is expressed as plaque forming units (pfu). O_3 = ozone environment, V = influenza virus infected, F = filtered air environment.

Interferon was released in the virus infected mice, and assays for the units of interferon in lung lavage fluids did not reveal any marked differences in the interferon titers among the groups of animals (Figure 8).

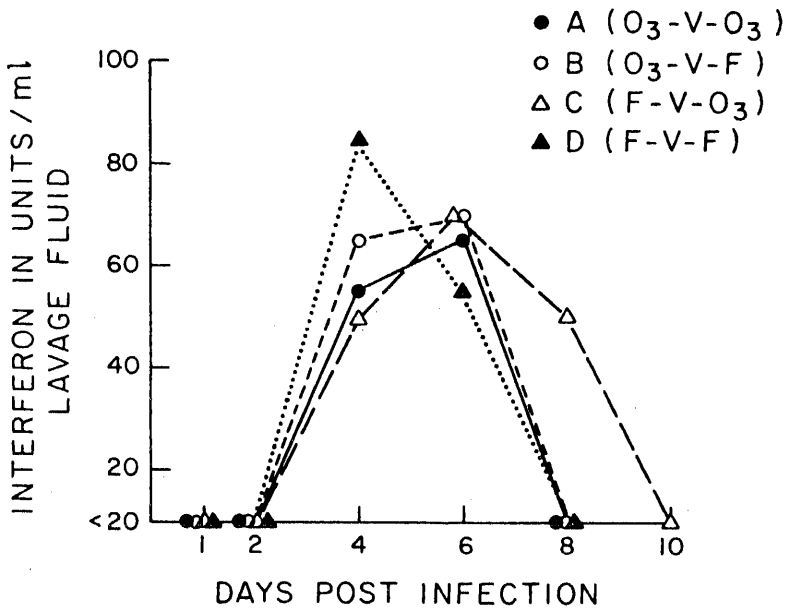


Figure 8. Units of interferon in lung lavage fluid of mice from Experiment #3. O_3 = ozone environment, V = influenza virus infected, F = filtered air environment.

Virus neutralizing antibodies appeared earlier in the serum and reached higher titers than was the case for neutralizing antibodies in the lung lavage fluids. The antibody response curves were quite similar among the 4 groups of animals (Figure 9).

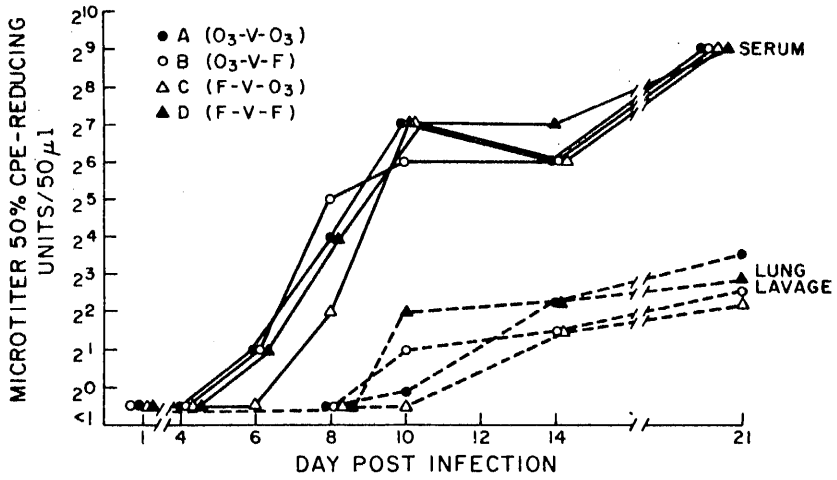


Figure 9. Assays for virus neutralizing antibodies in the serum and lung lavage fluids of mice in Experiment #3. Antibody titers were reported as cytopathogenic effect-reducing units (cpe). O_3 = ozone environment, V = influenza virus infected, F = filtered air environment.

Infected lung cells became involved in the synthesis of more virus. Since the virus is antigenic, the location of infected lung cells could be seen by adding fluorescienated antibodies, directed against the virus, onto sections of infected lung tissue (see Methods in Appendix II-B). The three following tables (Tables 9, 10, and 11) report results from an extensive examination of mouse lungs from Experiment 4. As noted previously, animals exposed to ozone, either before or after infection with influenza virus, experience a less severe influenza process. At this time we think that two effects are involved.

1. The damage to lung cells from continued ozone exposure is associated with replacement cells that are different from the

TABLE 9

SITES OF VIRUS REPLICATION BY FLUORESCENCE MICROSCOPY ON INFLUENZA-INFECTED MOUSE LUNG[1]

Day post infection	Exposure Protocol[2]	Airway determinations: Total airways observed	Airway determinations: Total positive airways	Airway determinations: Percent positive airways	Pleura determinations: Total pleural fields observed	Pleura determinations: Positive cells observed in pleura	Pleura determinations: Positive cells per 100 fields of pleura
2	O_3-V-O_3	477	1	0.2%	593	6	1.0
4		485	4	0.8%	578	111	19.2
6		459	4	0.9%	338	27	8.0
8		576	14	2.4%	937	15	1.6
2	O_3-V-F	447	18	4.0%	409	14	3.4
4		397	39	9.8%	434	72	16.6
6		603	27	4.5%	707	105	14.9
8		560	11	2.0%	719	16	2.2
2	F-V-O_3	458	18	3.9%	567	17	3.0
4		767	79	10.3%	625	29	4.6
6		700	28	4.0%	719	16	2.2
8		637	0	0.0%	635	7	1.1
2	F-V-F	494	77	15.6%	693	12	1.7
4		438	86	19.6%	402	48	11.8
6		553	27	4.9%	643	58	9.0
8		595	21	3.5%	676	32	4.7
Experiment totals		8,646			9,675		

[1] Data from Experiment 4.

[2] Symbols denote sequence of events (O_3 = ozone environment for 14 days, V = exposure to virus, F = filtered air environment for 14 days).

TABLE 10

SITES OF VIRUS REPLICATION BY FLUORESCENCE MICROSCOPY ON INFLUENZA-INFECTED MOUSE LUNG[1]

Day post infection	Exposure Protocol[2]	Alveolar determinations: Total alveolar fields observed	Antigen positive alveolar fields	Percent antigen positive fields	Observed antigen positive cells	Mean number of antigen positive cells per antigen positive field
2	O_3-V-O_3	1,565	42	2.7%	255	6.1
4		1,710	165	9.6%	2,807	17.0
6		2,390	300	12.6%	3,049	10.2
8		3,321	476	14.3%	1,497	3.1
2	O_3-V-F	1,505	115	7.6%	536	4.7
4		1,474	275	18.7%	3,038	11.0
6		3,232	1,075	33.3%	6,838	6.4
8		2,919	795	27.2%	2,252	2.8
2	F-V-O_3	1,473	221	15.0%	386	1.8
4		2,125	353	16.6%	1,051	3.0
6		3,405	561	16.5%	2,690	4.8
8		3,239	410	12.7%	1,049	2.6
2	F-V-F	1,958	195	10.0%	526	2.7
4		1,842	499	27.1%	2,179	4.4
6		2,422	890	36.4%	4,865	5.5
8		2,488	729	29.3%	2,683	3.7
Experiment totals		37,068				

[1]Data from Experiment 4.
[2]Symbols denote sequence of events (O_3 = ozone environment for 14 days, V = exposure to virus, F = filtered air environment for 14 days).

TABLE 11

CHI-SQUARE ANALYSIS OF PAIRED TESTS

FROM IMMUNOFLUORESCENCE DATA IN EXPERIMENT #4

Day post infection	Antigen positive airways			Antigen positive alveolar fields		
	Test	Paired chi-square statistic (df = 1)	P value	Test	Paired chi-square statistic (df = 1)	P value
2	A < D	77.69	<.0005	A < D	73.37	<.0005
	B < D	25.47	<.0005	B < D	5.61	<.025
	C < D	35.94	<.0005	C > D	20.07	<.0005
	A < B	16.71	<.0005	A < B	37.90	<.0005
	A < C	16.23	<.0005	B < C	40.32	<.0005
	B < C	-0-	NS*	A < C	145.17	<.0005
4	A < D	92.53	<.0005	A < D	177.47	<.0005
	B < D	15.74	<.0005	B < D	32.85	<.0005
	C < D	20.55	<.0005	C < D	64.24	<.0005
	A < B	38.14	<.0005	A < C	39.31	<.0005
	A < C	43.09	<.0005	A < B	53.93	<.0005
	B < C	.06	NS	C < D	2.52	NS
6	A < D	13.59	<.0005	A < D	378.28	<.0005
	B < D	0.10	NS	B < D	7.41	<.01
	C < D	0.57	NS	C < D	308.25	<.0005
	A < B	11.96	<.005	A < C	17.09	<.0005
	A < C	10.10	<.005	A < B	318.92	<.0005
	B > C	0.17	NS	C < B	251.52	<.0005
8	A < D	1.22	NS	A < D	193.83	<.0005
	B < D	2.64	NS	B < D	2.83	NS
	C < D	22.88	<.0005	C < D	244.60	<.0005
	A > B	0.28	NS	A < B	173.55	<.0005
	A > C	15.65	<.0005	A < C	3.93	NS
	B > C	12.61	<.0005	C < B	207.30	<.0005

*NS = Not significant

normal cells lining the airways. This alteration may be called "metaplasia", which refers to the change of one kind of tissue into another. The cells of this metaplastic membrane may have a changed status with respect to their ability for serving as host cells to the influenza virus.

2. Ozone, at the concentration used here (0.4 ppm), can inactivate influenza virus.

II. C. Evidence that metaplasia in the lung from ozone exposure changes the host susceptibility to influenza virus.

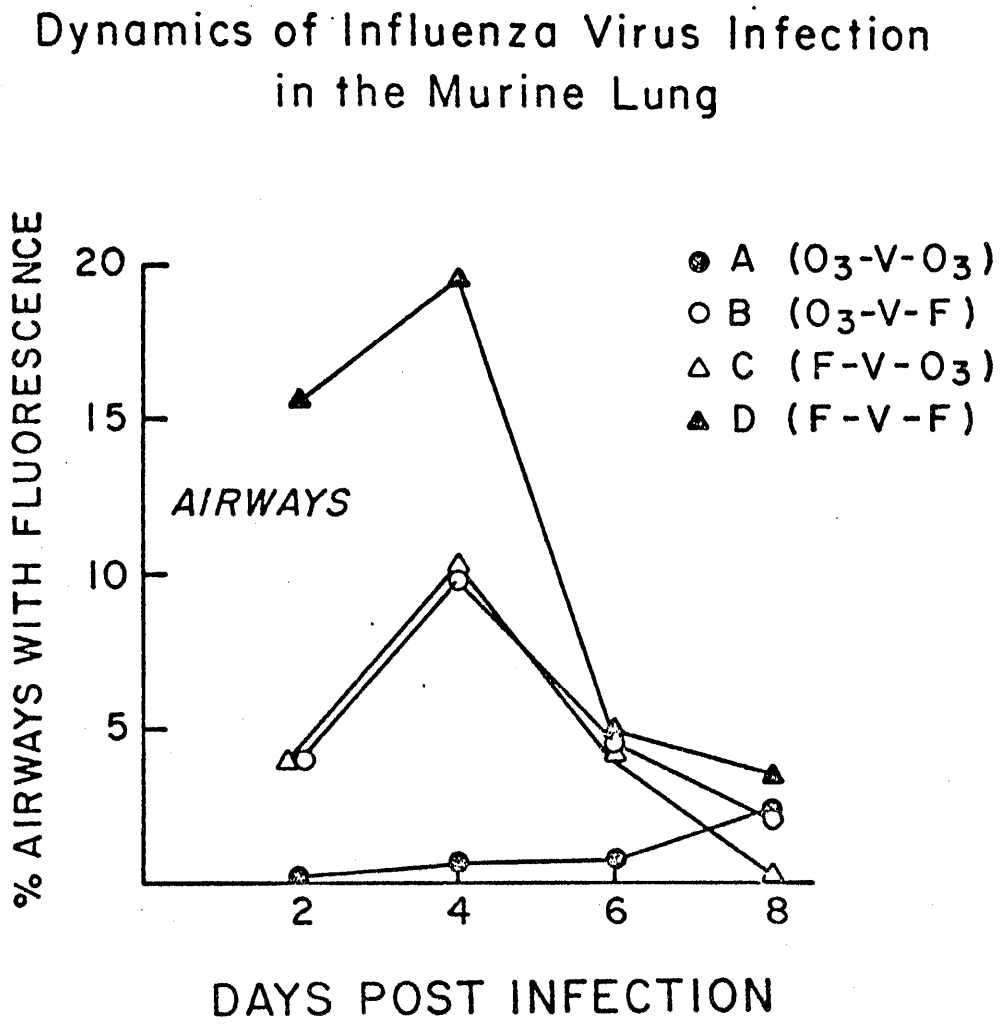
In these experiments Group A mice (O_3 - V - O_3) and Group B mice (O_3 - V - F) had been exposed to 14 days of continuous ozone (0.4 ppm). The metaplastic changes from the deleterious ozone effects would result in loss of cilia on lining epithelial cells, replacement of damaged airway lining epithelium with a flattened cellular form, and changes in some intracellular enzymes. In addition, there is evidence of replacement of Type I pneumonocytes with Type II pneumonocytes in the alveolar tissue. It is still uncertain how many ways this abnormal lung surface may change lung function. The changes indicate a form of partial lung repair in the face of a persisting injurious irritant. The influenza virus constitutes a parasite which has become adapted to normal lung cells for replication. In ways not yet determined, the ozone modified respiratory cells may not function for influenza virus in a manner identical with normal cells.

The experiments discussed here indicate that there is less mortality when the lungs are modified from ozone than is seen when mice are in a filtered air environment (Group D). Also, host modification is associated with a lessened degree of virus replication in lining cells of the airways. Another difference concerns the distribution of virus in ozone altered lungs (Figures 10 and 11). Virus density tends to be greater in restricted areas, but the virus is not so diffusely disseminated throughout lung parenchyma. (These observations are made from a limited amount of data at this time.)

II. D. Inactivation of influenza virus by ozone.

In Group C (F - V - O_3) the animals were not modified by ozone exposure. Instead, the mice were first infected with aerosolized virus and then the inhalation of ozone began. A marked reduction in mortality was seen in these mice when compared with Group D animals (F - V - F). Group A (O_3 - V - O_3) and Group C (F - V - O_3) inhaled ozone after virus infection and had reduced mortality. The airways of Group C were more heavily infected than Group A (Because Group C mice were not modified from ozone exposure?).

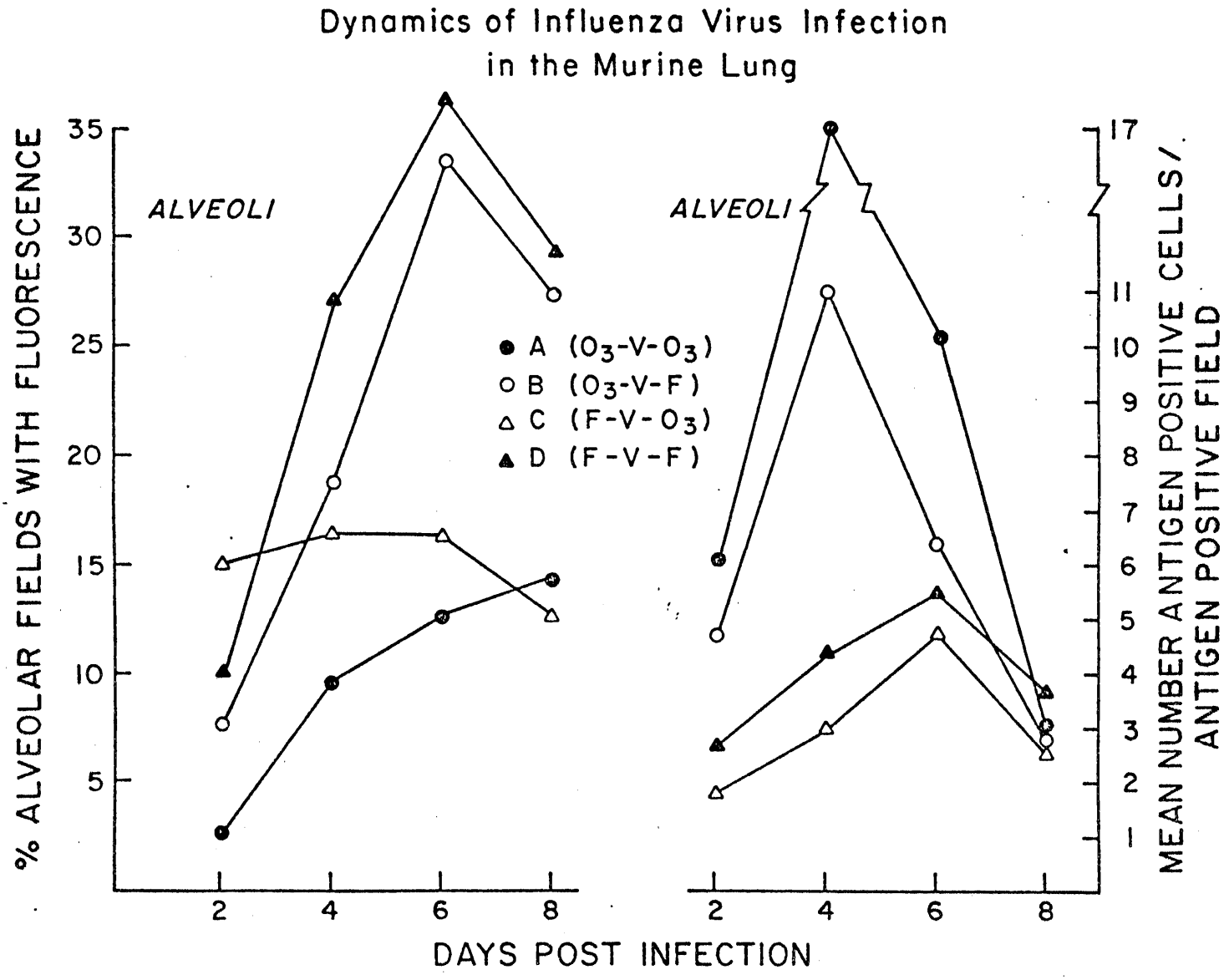
Figure 10



It was also observed that the virus in alveolar tissue of Group C mice tended to be disseminated. This was similar to the virus distribution in Group D mice, which, likewise, were not modified by ozone.

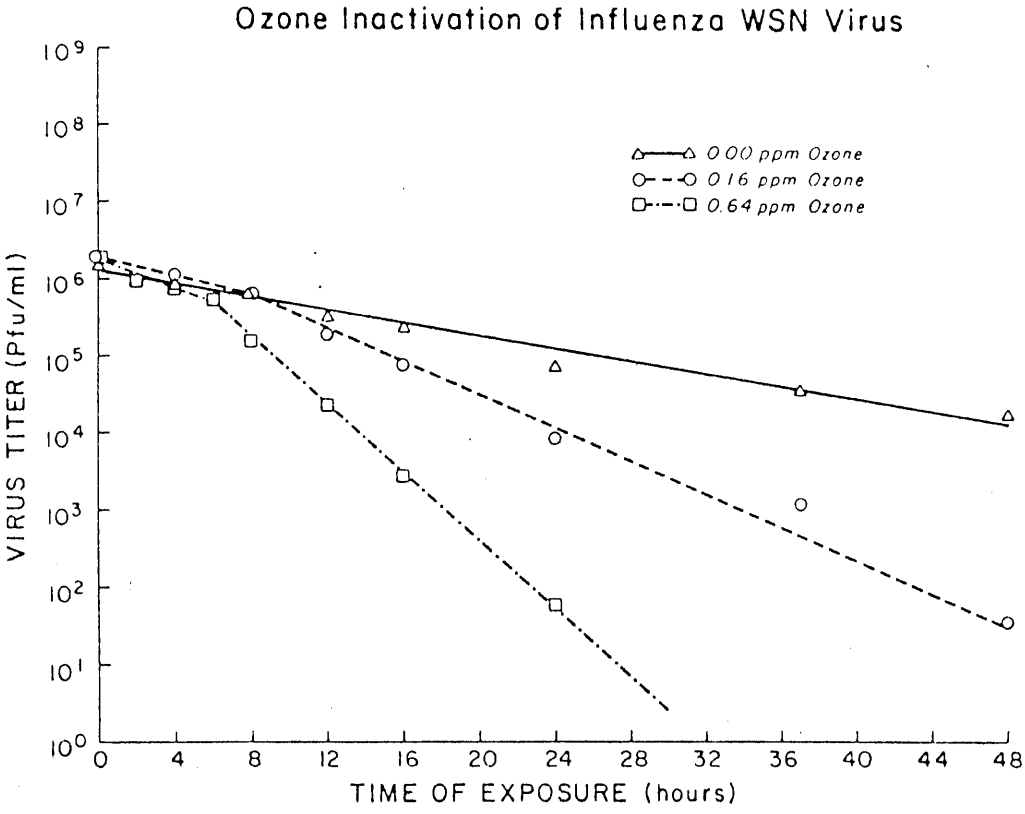
If we are correct, that two phenomena come into play with respect to altering influenza from environmental ozone, then one can see why the highest survival rate occurred in Group A mice. Those animals were ozone exposed before and after infection, which would render them ozone modified prior to infection and subject to ozone inactivation of virus after infection.

Figure 11



The proposal that ozone inactivates influenza virus is more than first conjecture. In a study supported by other sources, our laboratories have devised an instrument which creates an in vitro system for the study of ozone effects on mammalian cell cultures and viruses. Two manuscripts describing this work have been submitted for publication (attached). The first paper describes the instrument. (The CARB is cited for support since it was timely to do the manuscript preparation during this project period.) The second paper reports the effects of ozone on inactivation of five groups of viruses. Three enveloped viruses, including influenza virus, were susceptible to ozone inactivation, while two nonenveloped viruses (polio virus type I and canine hepatitis virus) were resistant. The graph (Figure 12) shows results with influenza virus at 0.16 ppm and 0.64 ppm of ozone. In the mouse experiments considered here, ozone was presented continuously in a concentration of 0.4 ppm. Following the initial infection with aerosolized virus, there would be attachment and then penetration of virus into cells.

Figure 12



At the time newly synthesized virus was ready to emerge by budding off the surface of infected cells, it would encounter ozone in the airways. It is our supposition that ozone in such a concentration can inactivate emerging virus, which must achieve transit along the airway before finding new susceptible host cells for further replication. The effect of this could markedly slow the disease process and allow time for immune responses to arise and begin neutralizing virus with antibodies and destroying virus infected cells with cytotoxic T-lymphocytes.

Our present evidence suggests that influenza virus is uniquely susceptible to inactivation by ozone because of its envelope. The envelope contains a lipid bilayer similar to mammalian cell membranes, which makes it subject to oxidation damage by free ozone. The data on non-enveloped viruses shows them to be essentially resistant to ozone. This suggests that other viruses of the respiratory tract would not be subject to the loss of viability encountered with the influenza virus.

APPENDIX

I-A Materials and Methods for Enhancement of Allergic Lung Sensitization by Inhalation of Ozone

Animals

Animals used were a specific pathogen-free (SPF) line of Swiss-Webster mice (Hilltop Lab Animals, Inc., Scottdale, PA). The animals were monitored by the supplier for a variety of infectious agents including Mycoplasma, Pasteurella, Klebsiella, and the pneumonia virus of mice. Mice were acclimatized to their new environment for a few days while housed in an isolation room with fifteen air exchanges per hour at 22°C.

Ozone Exposure

Mice were exposed to ozone in modified fiberglass environmental units (Germ-free Labs., Inc., Miami, FL). A silent electrical discharge ozone generator was used to produce ozone from bottled oxygen. Ozone was admitted into the chamber after mixing with filtered room air. Air supplied to each chamber, and the exhaust air, was passed through C-B-R filters (Mine Safety Appliance Corp., Pittsburgh, PA), with airflow through the chamber set at fifteen volume changes per hr. The ozone concentration of 0.4 or 0.64 ppm (Experiments A, B, and C) was continuously monitored with an ozone meter (Mast Development Co.). The ultraviolet photometric ozone analyzer (Dasibi Environmental Corporation, Glendale, CA) was used in Experiments D and E. The ozone exposure was continuous, except for the interruption to clean cages which required opening chambers to ambient air for approximately 15 min each day. Control animals were housed in identical chambers and maintained in an environment of filtered ambient air.

Lung Lavage Samples

Pulmonary lavage was performed on anesthesized (pentobarbital sodium) mice which were exsanguinated by severing the renal artery. A catheter was inserted into the trachea and 1-ml volumes of 0.9% sodium chloride solution (PSS) were infused and withdrawn three times for a total infusion of 3 ml, with approximately 2.6 ml recovered. The lavage procedure immediately followed the blood collection, and was completed within 5 to 10 min of each animal's death. Fluids withdrawn from the lungs of a group of mice were pooled and chilled in an ice bath. An aliquot was removed for microbiological analysis and the remainder was preserved by thimerosal addition (Ruger Chemical Co., Irvington, NJ) at a final dilution of 1:10,000 and centrifuged to remove cells. Lung lavage samples pooled from eighteen mice were then concentrated about 50X by vacuum dialysis with the Collodion Bag Apparatus (Schleicher and Schuell, Keene, NJ). Concentrated samples were approximatley 1 ml in volume. They were centrifuged at 500 X g for 30 min at 5°C to remove sediment, and aliquots were taken for protein estimation by the method of Lowry et al. Concentrated fluids from normal mice contained approximately 0.3 mg of protein per mouse. Lung lavage fluids and serum samples were stored at -20°C.

Immunodiffusion

Single radial diffusion tests were run in rectangular dishes containing twenty-four antigen wells of a size that accommodated 5 µl volumes of antigen. The preparations were incubated for 48 hr at room temperature, washed, dried, and stained. Samples were analyzed in triplicate to obtain the precipitin ring diameters. Standard curves were prepared for immunoglobulin assays from an ascites fluid standard (Meloy Laboratories) and for albumin from a mouse serum albumin preparation (Cappel Laboratories).

Aerosolization of Ovalbumin

At intervals the mice were exposed to nebulized ovalbumin (OA) in a Tri-R Airborne Infection Apparatus (Tri-R Instruments, Inc., NY). The nebulizer was designed to yield aerosol droplets of less than 3 µm (range of 0.5 to 3 µm). About 45 min elapsed between the time that animals were taken from the air pollution chambers and then brought into contact with the antigen. Mice from the various groups ($\simeq$ 50 animals) were placed in the chamber and were exposed for 30 min to nebulized OA (1% or 2% solution in sterile distilled water). The relative humidity in the chamber was approximately 50 percent at 22°C.

Sensitization of Positive Control Mice

Mice were sensitized to OA by two intraperitoneal (IP) injections, at an interval that varied from 19 to 35 days in the different experiments. The injections were 1 ml in volume containing 1 µg of OA and 1 mg of alum precipitate.

Systemic Anaphylaxis in Mice Injected with Ovalbumin

Testing for anaphylactic sensitization was performed 6 to 8 days following the last sensitization with aerosolized OA. The mice were injected intravenously (IV) in a tail vein with 2 mg of OA in 0.1 ml of distilled water. They were carefully observed over a 2-hr period for evidence of anaphylactic shock, and recovery or death from the reaction[13].

Air Pollution Experiment #38

(AP-38)

April 16, 1980

Objectives:

1. To determine the allergy enhancing effects of ozone (0.16 ppm) and O_3 + H_2SO_4 (0.5 mg/m^3).
2. To determine the effect of antigen dose on allergic response.
3. To determine the effect of B. pertussis adjuvant for IgE responsiveness.
4. To test for serum IgE by heterologous PCA tests.
5. To examine lung and spleens for IgE producing cells.
6. To associate anaphylactic shock with the number of IgE cells in the lungs.
7. To test responses to injected OA in O_3 exposed mice.

Animals:

5 week-old male SPF Swiss Webster mice from Hilltop Laboratories.

Exposure Chambers:

Mice will be housed in 3 stainless steel chambers as follows:

Ozone (0.16 ppm) - 130 mice + 20 mice (no OA aerosol)

Ozone (0.16 ppm) + H_2SO_4 (0.5 mg/m^3) - 130 mice

Ambient air - 130 mice

The remaining mice which includes positive and negative controls will be housed in Isolation building (IS-6).

Procedure:

1. At each of the 2 shocking series, mark the mice that go into shock. After the shocking injection, fix lungs and spleens from 3 mice that were shocked and 3 that were not shocked. (Make the usual H&E and frozen preparations.)
2. Serum collections for IgE tests (Heterologous PCA) - Seven days after each aerosolization, bleed 10 mice (5 with adjuvant and 5

without adjuvant) from orbit - return them to continue to the end of experiment.

APRIL	DAY #	DAY OF WEEK		
16	0	Wed	Place in chambers - bleed mice	
17	1	Thur		
18	2	Fri		
19	3	Sat	Aerosol	
20	4	Sun	Remove & Aerosol	
21	5	Mon		
22	6	Tues		
23	7	Wed		
24	8	Thur		
25	9	Fri		
26	10	Sat		
27	11	Sun		
28	12	Mon	Return to chambers	
29	13	Tues		
30	14	Wed		
MAY				
1	15	Thur		
2	16	Fri	Remove and Aerosol	bleed mice
3	17	Sat		
4	18	Sun		
5	19	Mon		
6	20	Tues		
7	21	Wed		
8	22	Thur	Return to chambers (Hold 60 mice each chamber in ambient)	
9	23	Fri	Shock	
10	24	Sat	Process shock lungs	
11	25	Sun		
12	26	Mon	Remove & Aerosol	+ Bleed mice
13	27	Tues		
14	28	Wed		
15	29	Thur		
16	30	Fri		
17	31	Sat		
18	32	Sun		
19	33	Mon	Return to chambers	
20	34	Tues		
21	35	Wed		
22	36	Thur	Remove & Aerosol	+ Bleed mice
23	37	Fri		
24	38	Sat		
25	39	Sun		
26	40	Mon		
27	41	Tues		
28	42	Wed		
29	43	Thur		

MAY	DAY #	DAY OF WEEK		
30	44	Fri	Shock	+ Bleed mice

Shock days - 30 mice with adjuvant
30 mice without adjuvant + 10 ea positive & negative mice
+ 10 O_3 exposed - no aerosol

I-C Materials and Methods used for Studying the IgE-Containing Cells in Lungs.

Animals

Tissues were obtained from mice in Experiments A, B, & C for these studies. The methods for aerosolizing the allergen, exposure to ozone, and immunizing positive control mice were described in Appendix I-A.

Tissue Preparation. Following the final aerosolization with OA, mice were anesthetized with pentobarbital sodium and terminally bled from the renal artery. Blood was collected for serum analysis. An inert, water-soluble supporting medium for frozen tissues (Tissue-Tek, Labtek Products, Miles Laboratories, Naperville, Ill.) was infused into the lungs via the trachea. The trachea was tied off to hold the fluid in the expanded lungs. The left lobe was removed and placed in 10% neutral buffered formalin for 3 h at 4°C. It was then cut longitudinally down the main bronchus and the ventral portion was placed into 30% sucrose at 4°C for approximately 18 h to leach out the formalin. The lung tissue was then frozen in Tissue-Tek and stored in liquid nitrogen. Sections of 6 μm thickness were cut on a cryostat. These serial sections of the left lung were oriented longitudinally through the lobar bronchus and placed on acid-cleaned and gelatin-subbed slides. The sections were dried, acetone-fixed for 10 min and stored at -20°C. They were usually stained and examined within a week.

Antiserum to Mouse IgE. It was necessary to prepare goat anti-mouse IgE serum. Details of the procedure will be presented elsewhere. Mouse IgE for immunization of the goats was obtained from the serum and ascites fluid of Swiss-Webster mice following immunization with OA. The mice received a primary intraperitoneal (IP) injection containing 1 μg of OA in 1 mg of alum precipitate. A secondary injection was made 3-4 weeks later. Ascites was induced by weekly IP injections of complete Freund's adjuvant (0.2 ml). Serum and ascitic fluid from immunized mice were precipitated with 50% saturated ammonium sulfate and then chromatographed by gel filtration on Biogel A-5m (Bio-Rad Laboratories, Richmond, Calif.). Fractions demonstrating IgE activity by passive cutaneous anaphylaxis (PCA) tests on rat skin were pooled and used to immunize goats according to the protocol of Lang et al. Antiserum collected from the goats was precipitated with 50% saturated ammonium sulfate and exhaustively absorbed with mouse serum proteins of the SJL strain coupled to cyanogen bromide-activated beads (Sepharose 4B, Pharmacia, Uppsala, Sweden). The absorbed sera were nonreactive in micro double-diffusion tests against the following mouse myeloma proteins: IgA, IgG_1, IgG_2, IgG_3, and IgM (Meloy Laboratories, Springfield, VA). The presence of antibodies to IgE in the absorbed goat sera was demonstrated by PCA inhibition tests for mouse IgE on rat skin. Prior incubation of IgE-containing (PCA-positive) mouse serum or ascitic fluid with absorbed goat antiserum neutralized its ability to elicit a positive PCA test.

Specificity of the Anti-IgE Conjugate. The anti-IgE conjugate was considered to be epsilon chain-specific following its absorption with normal

serum of the SJL mouse strain to remove antibodies to L chains and heavy chain classes other than IgE. In addition, background staining of lung stroma was greatly diminished by absorption with collagen. The conjugate showed no precipitation lines against IgM, IgG_1, IgG_2, IgG_3, and IgA on template micro double-diffusion tests.

Positive control tests included the detection of some cells with fluorescing cytoplasm in preparations from the mediastinal lymph nodes and spleens of mice immunized IP with OA. Membrane staining of rat mast cells was seen following their incubation with IgE-containing mouse serum. Blocking control tests were performed by incubating the mast cells with either normal goat serum or unconjugated goat antiserum prior to the addition of conjugated goat anti-IgE serum. Fluorescence was greatly diminished by the unlabeled goat anti-IgE. As will be detailed in this report, the numbers of fluorescing cells in lungs correlated positively with levels of sensitization as detected by anaphylactic reactivity. In preparations where IgM-, IgG-, and IgA-containing cells were delineated by rhodamine fluorescence, there was no evidence of double staining from the fluorescein-labeled anti-IgE reagent.

Negative control tests included lung and spleen sections from mice of the SJL strain, cultures of mouse L cells (fibroblasts), and preparations of macrophages and other cells lavaged from the peritoneal cavities of normal Swiss-Webster mice. Also, the lung sections from several normal control mice of the Swiss-Webster line contained no cells reacting with the anti-IgE conjugate.

Localization and Enumeration of IgE-Containing Cells. The goat anti-mouse IgE sera were found to contain antibodies to collagen, which were removed by absorption with a combination of bovine collagen and mouse skin collagen. The antisera were conjugated with fluorescein isothiocyanate[15] and the direct fluorescent antibody method was used to visualize IgE-containing cells. Positive cell control preparations consisted of acetone-fixed droplets of rat mast cells sensitized with mouse IgE, and mediastinal lymph node cells obtained from mice immunized by the IP route with OA in alum. Negative cell controls consisted of lung and spleen sections from SJL mice, a genetically low responding strain for IgE production.

The anti-IgE conjugate was diluted in phosphate-buffered saline (PBS), pH 7.2, and applied to the cell or tissue samples for 1 h at 37°C in a moist chamber. Staining was followed by three 10-min rinses in PBS. The sections were covered with mounting medium (1:1 glycerol/PBS) and a cover slip. Stained sections were examined on a Zeiss microscope with epi-illumination at magnifications of X 160 and X 400. The total number of fluorescent cells was counted at a magnification of X 160 on a section from each lung. Cells were classified according to their location; i.e. airway-associated, and within lung parenchyma.

A double fluor labeling technique was used to further demonstrate the specificity of the anti-IgE conjugate. In an indirect fluorescent method goat anti-sera specific to mouse heavy chains (gamma 1, gamma 2, mu, and alpha) (Meloy Laboratories) were used as the primary reagent on lung sections. Rabbit anti-goat IgG (Cappel Labs., Cochranville, PA) conjugated

with rhodamine was used as the secondary reactant. The indirect fluorescent procedure detected different cells from those reacting with the goat anti-mouse IgE when the tests were applied to the same section of lung or to serial sections.

Airway Measurement and Planimetry. Following the examination of IgE-containing cells, the lung sections were fixed in 10% neutral buffered formalin for 30 min, stained with a 0.05% aqueous solution of methylene blue for 1 h, rinsed, dried and mounted under a cover slip. An image of each lung section was magnified at X 40 with a Leitz projecting microscope. The outlines of the tissue and airways were traced onto paper. The total airway distance was determined by tracing over the perimeter of each airway with a map measure (Minevra, Switzerland), thus accumulating a distance convertible to millimeters of airway on the section. The area of lung tissue on the drawing was measured with a Hruden planimeter (Ann Arbor, MI), and then converted to the actual area on the section.

Counterimmunoelectrophoresis. Counterimmunoelectrophoresis (CIE) tests were performed as described by Crowle[16]. Undiluted serum was electrophoresed opposite varied dilutions of OA (0.001 through 0.1 mg/ml). Test wells contained 25 µl.

Homologous PCA. Homologous PCA tests were performed on mouse skin to detect IgG_1 homocytotropic antibodies[17]. Tests were run in triplicate and volumes of 0.05 ml of undiluted serum were injected intradermally (ID). Following a 2-hour latent period 1 mg of OA and 1 mg of Evans blue dye in 0.1 ml of PBS was injected IV.

Heterologous PCA. For the detection of mouse IgE in serum and ascitic fluid, serial dilutions of samples were tested on rat skin[17]. ID doses of 0.1 ml were injected, and after a 24-hour latent period, an IV injection of 5 mg OA with 5 mg Evans blue dye was made. Positive control sera were included in the tests.

II-A Materials and Methods for Studying Pneumonia Induced by Influenza Virus

Virus

Influenza A_0 virus (WSN) was propagated in Madin-Darby bovine kidney (MDBK) cells as described by Coppin[18].

Mice

Male Swiss-Webster mice (10 to 13 weeks old) were used (Horton Lab, Oakland, CA) except for the specific pathogen-free mice (Hilltop Lab Animals, Inc., Scottdale, PA) which were used in the trial producing 16% mortality and for the studies identified as Experiments 1, 2, 3, and 4. Mice were housed in an isolation room with 15 air exchanges per h at a temperature of approximately 22°C.

Aerosol Exposure of Mice to Influenza Virus

Groups of mice (usually 65) were exposed to nebulized influenza virus in a TRI-R airborne infection apparatus (TRI-R Instruments, Inc., New York, NY) for 30 min. The relative humidity in the exposure chamber was approximately 50% at 22°C.

Virus Assay

Lungs from six mice for each sampling day were harvested aseptically, and a 10% lung homogenate was prepared in Eagle medium. The tissue suspensions were centrifuged at 400 X g for 15 min, and the supernatant fluid was assayed for infectious virus in MDBK cells by the plaque method under a 1.0% agar overlay.

Collection of Lung Lavage and Serum Samples

Pulmonary lavage was performed on anesthesized mice (pentabarbital sodium), which were bled out by severing the renal artery. Blood was collected for serum analysis. The lavage procedure immediately followed blood collection and was completed within 5 to 10 min after death. The lung fluids from groups of 18 mice were pooled and chilled. The samples were centrifuged to remove cells and then concentrated about 50 times by vaccum dialysis with a collodion bag apparatus (Schleicher & Schuell Co., Keene, NH). Concentrated fluids from normal lung lavages contained approximately 0.3 mg of protein per mouse. The pooled lung lavage fluids and serum samples were stored at -20°C.

Immunodiffusion

Quantitative assays for proteins in the lung lavage fluids and sera were performed with the single radial diffusion method. Goat antisera to the heavy chains of mouse IgG_1, IgG_2, IgA, and IgM (Meloy Labs, Springfield, VA) or anti-mouse albumin (Cappel Labs, Inc., Cochraneville, PA) were incorporated into 1% agarose. Tests of the antisera against mouse serum indicated the purity of the reagents.

Single radial diffusion tests were run in rectangular dishes and analyzed in triplicate to obtain the precipitin ring diameters. Standard curves were prepared for the immunoglobulin assays from an ascites fluid standard and for albumin from a mouse serum albumin preparation.

Interferon Assay

Pooled bronchopulmonary lavage fluids were assayed for interferon activity by the method for vesicular stomatitis virus plaque reduction in L cells[19]. Interferon units were expressed as the reciprocal of the dilution causing 50% plaque reduction. A standard reference mouse interferon (catalog no. G00-90A-511; National Institutes of Health) was included in each assay. One unit of interferon in our assay was equivalent to two units of the reference interferon.

Identification of the virus-inhibiting substance in the bronchopulmonary lavage fluids as interferon was based on the following criteria: host species specificity, trypsin sensitivity, non-dialyzability, stability at pH 2.0, failure to sediment at 100,000 X g, and inhibition by actinomycin D.

Neutralizing Antibody Assay

Neutralizing antibody titers in pooled lung lavage fluids and pooled sera were determined by the 50% plaque reduction technique.

Indirect Fluorescent Antibody Assay

Immunoglobulin class-specific influenza virus antibodies in lung lavages were demonstrated by the indirect fluorescent antibody method[20]. Slide cultures of BHK cells were infected with influenza virus and fixed with acetone. Concentrated lung lavage samples were diluted with saline, and each dilution was applied to duplicate slide cultures. The slides were incubated at 35°C for 1 h and washed twice with phosphate-buffered saline (pH 7) for 10 min. The slides were stained with fluorescein-conjugated antisera prepared in goats against the heavy chains of mouse IgG_1, IgG_2, mounted in 25% glycerin in phosphate-buffered saline (pH 7.6), and examined with a Zeiss microscope using epi-illumination.

Sectioning and Fixing Lung Tissues for Study of Antibody Containing Cells

Mice were anesthetized with pentabarbatal sodium and exsanguinated. Lungs were infused in situ with an inert, water soluble supporting medium for frozen tissues (Tissue-Tek, Labtek Products, Miles Laboratories, Naperville, IL). Approximately 0.7 ml of Tissue-Tek was infused through the trachea, which was then tied off to prevent escape of the viscous fluid. The left lung was cut longitudinally down the main bronchus, frozen in Tissue-Tek, and stored in liquid nitrogen.

Lungs sections of 6μm thickness were cut on a cryostat. Serial sections of the left lung, oriented longitudinally through the lobar bronchus, were placed on clean slides. The slides were dried, acetone fixed for 30 min, and stored at minus 20°C until they were stained and examined.

Enumeration of Antibody Containing Cells

Fluorescein conjugated antisera to the heavy chains of mouse immunoglobulins were used to detect antibody forming cells in lung tissue by the direct staining technique. Antisera were produced in goats from mouse myeloma proteins (Meloy labs, Springfield, VA).

The conjugated antisera were diluted in phosphate buffered saline (PBS, pH 7.2) and tested against mouse spleen cells as positive controls. Negative controls were obtained from normal lung tissue and preparations of alveolar macrophages. When assaying for antibody forming cells in lung tissue, the conjugated anti-immunoglobulins including anti-IgA, anti-IgM, and anti-IgG were applied respectively to the first, second, and third serial sections on a slide. Slides were incubated for 1 h at 37°C with the conjugates. Sections were washed and then covered with mounting medium (1:1 glycerol and PBS, pH 8.3) and a coverslip.

Examinations were made on a Zeiss microscope using eip-illumination. A 16 X objective and a 10 X eyepiece were used to photograph regions of the lung used for cell counting. Counts were made on 18 photographed fields per section comprising an area of 209 mm^2. The 18 microscopic fields were designated as a Lung Unit (LU), which was located along a 4.0 mm section of the lobar bronchus, and the adjacent tissue, out to the pleural surface. Counts were made from three tissue locations:

(a) Six fields along the bronchus, with "membrane related cells" counted as those located in the lamina propria. By definition, membrane related cells were within photographed fields containing the major airway. Antibody-containing cells that were clearly located in adjacent alveolar tissue were scored as lung parenchyma related cells.

(b) Six fields in areas of cellular hyperplasia or infiltration. These regions included consolidated lung lesions and areas of lymphoid cell expansion around blood vessels and at branch points of the airways.

(c) Six fields of alveolar tissue that had normal, or near normal, cellularity. Cells counted showed cytoplasmic staining. Microscopic fields were photographed on Tri-X panchromatic film (Eastman Kodak Co., Rochester, NY). To facilitate counting, the 35 mm negatives were projected on a screen containing grid lines.

II-B Materials and Methods for the Study of Ozone Effects on Influenza

Scoring of Lesions

Stained lung sections were projected at X 20 magnification with a Leitz projecting microscope. A drawing was made of the section outline, the airways, and areas of consolidation (lesions). A system for scoring lesions according to location was devised. Individual areas of consolidation (regions of cellular infiltration were assigned numerical values on the following basis:

1 = Lesion in contact and associated with the bronchus.

2 = Lesion in the central parenchyma, but showing an extension to the bronchus.

3 = Lesion entirely in the central parenchyma.

4 = Lesion in the central parenchyma, but showing an extension to the pleural surface.

5 = Lesion extending along the pleural surface.

The mean of the accumulated numbers for the section represented a score, ranging from 0 to 5, which described the lesion distribution.

Preparation of Lungs for Detection of Virus Location

The right, apical lobe of each mouse was step-sectioned in a cryostat (Harris International Equipment, Needham Heights, MA) at a thickness of 4 μm. Sections were taken from all areas of one entire lobe leaving at least 80 μm and not more than 120 μm between sections so as to avoid sampling of the same area of the lung lobe in adjacent tissue sections.

Preparation of Rabbit Antiserum to Influenza Virus

Adult New Zealand white rabbits were given four 1.0 ml inoculations of concentrated influenza virus that had been propagated in the allantoic cavities of 11-day-old chick embryos. Allantoic fluid containing influenza virus (1.35×10^7 pfu/ml) was mixed in equal parts with Freund's incomplete adjuvant. Inoculations were administered subcutaneously at intervals of 7 to 10 days. Serum was collected 7 days after the last injection.

Preparation of Fluorescent Antibody Conjugate

The globulin fraction of the rabbit antiserum to influenza virus was precipitated with $(NH_4)_2SO_4$ and the protein content was determined by the method of Lowry. The antiserum was labeled with fluorescein isothiocyanate by the method of Clark and Shepard. The conjugate was dialyzed overnight against 0.85% NaCl and passed through Sephadex G-25 (Pharmacia, Uppsala, Sweden) in 0.01 m phosphate-buffered saline (PBS) at pH 7.3. The fluorescent conjugate that was used to stain macrophages was absorbed with freshly disrupted L cells (mouse fibroblasts), and conjugate applied to lung sectons was absorbed with freshly disrupted mouse lung tissues.

Immunofluorescent Method

Fluorescein-conjugated antiserum to influenza viral antigens was used to detect cells containing viral antigens by direct immunofluorescence. A drop of a 1:32 or 1:64 dilution of the conjugate was applied directly to each acetone-fixed, frozen lung section or macrophage preparation. Slides were then incubated in a humidifed chamber at 37°C for 1 h. The preparations were rinsed and washed in three changes of 0.01 M PBS (pH 7.3), with a final washing in double-distilled water. Slides were air-dried and mounted with a mixture of equal parts of glycerol and 0.01 M PBS (pH 8.3)

and examined in a Zeiss fluorescence microscope (Carl Zeiss, New York, NY) using epi-illumination.

Control tests with the fluroescein-labeled conjugate to influenza virus gave positive staining with influenza virus-infected MDBK cells and negative reactions when uninfected MDBK cells were used. The conjugate did not stain heterologous antigen as shown by tests with MDBK cells infected with vesicular stomatitis virus. Inhibition testing with unlabeled antiserum to influenza virus gave negative or greatly diminished staining when the conjugate was added. Normal serum did not demonstrate inhibitory activity.

When the conjugate was absorbed with heterologous antigens (mouse L cells or normal mouse lung tissue), the reagent retained its activity against viral antigens.

Determination of Sites of Virus Replication

A minimum of 80-100 tissue sections for each of the four-environmental groups was observed on each sampling day. Tissues were initially scanned at 160 X magnification in an S-shaped pattern and the number of airways with and without fluorescence in the lining epithelium were counted. Antigen-positive airways were defined to be airways with specific fluorescence in at least 40% of the visible lining epithelium. The percentage of antigen-positive airways was calculated for each group according to the sampling day. The same tissue sections were then scanned in an S-shaped pattern at X 400 magnification. The alveolar area of each microscopic field was 0.159 mm^2. Each alveolar field which contained at least one fluorescent cell was defined as an antigen-positive field. The number of antigen-positive fields counted for each group according to sampling day was expressed as a percentage of the total number of alveolar fields examined. The number of alveolar cells expressing fluorescence in each antigen positive field was counted without differentiating between cell types. The mean number of antigen positive cells per antigen positive field was calculated. The pleura was scanned at X 400 magnification and the number of fluorescent cells expressed as the mean number of antigen positive cells per 100 observed fields of pleura.

Statistical Analysis

Four-way and paired χ^2 tests were used to analyze data on antigen positive fields of alveolar parenchyma. An analysis of variance was performed on data from determinations of the mean number of antigen positive cells per antigen positive field.

EXAMPLE PROTOCOL

EFFECTS OF OZONE ON INFLUENZA VIRUS INFECTION

Objectives: To determine the effect of 0.4 ppm ozone on the pathogenesis of influenza virus infection in mice. Parameters to be measured are mortality, virus titers in lungs, neutralizing antibody titers in lung lavages and sera, interferon titers in lung lavages and sera, and the cells of the lungs infected with viruses determined by immunofluorescence.

Virus: Influenza WSN strain.

Animals: CRD-free, Swiss Webster, male mice; 10 weeks old, supplied by Hilltop.

Exposure Protocol:

Group	# Mice	First 2 wks in 0.5 ppm O_3	First 2 wks in Ambient Air	Virus Exposure	Second 2 wks in 0.5 ppm O_3	Second 2 wks in Ambient Air
A	120	2 wks	-	+	2 wks	-
B	120	2 wks	-	+	-	2 wks
C	120	-	2 wks	+	2 wks	-
D	120	-	2 wks	+	-	2 wks
E	6	2 wks	-	-	-	-

TOTAL MICE: 486

Chamber Requirements: One large ozone chamber for ozone and ozone-virus mice, one large ambient chamber for ambient mice, as per exposure protocol above.

Procedures: As indicated in the attached schedule, mice will be removed from the chambers and brought to Haring Hall. Lung lavages will be collected separately and serum samples will be collected separately or pooled as indicated. Whole lungs will be collected for virus assay or embedding in tissue tek.

Time for Starting: 9 a.m. (i.e. mice will be at 2014 Haring Hall)

PROTOCOL CONTINUED

Days After Virus Exposure	Lung Homog & Serum* (Pooled) A	B	C	D	Lung Wash & Serum (IND.) A	B	C	D	Lung Whole Mt & Serum* (Pooled) A	B	C	D	Help Required
1	2	2	2	2	4	4	4	4	4	4	4	4	S W L
2	2	2	2	2	4	4	4	4	4	4	4	4	S W D L J Z
4	2	2	2	2	4	4	4	4	4	4	4	4	S W D L J Z
6	2	2	2	2	4	4	4	4	4	4	4	4	S W D L J Z
8	2	2	2	2	-	-	-	-	4	4	4	4	S W D L
10	-	-	-	-	4	4	4	4	4	4	4	4	S W L
14	2	2	2	2	4	4	4	4	4	4	4	4	S W D L J Z
21	2	2	2	2	4	4	4	4	4	4	4	4	S W L

Pooled Serum: For serum Neutralizing Ab.

Individual Serum: For serum IF

Lung Washes: For L.W. IF and LW Neutr. Ab.

Lung Homogenates: For virus titration

Lung Whole mounts: For FA - NOTE: All lungs to be perfused with Tissue-tek before removing from the animal.

NOTE: 6 O_3 only mice to be used for lung whole mount and Serum-IF collection as controls.

*S: Sharon
W: Judy
D: Dave
L: Lata
J: Jack
Z: Zee

REFERENCES

1. Shy, C. M., Health Hazards of Sulfur Oxides. American Lung Association Bulletin, p. 2-7, March, 1977.

2. French, J. G., G. Lowrimore, W. C. Nelson, J. F. Finklea, T. English, M. Hertz. The Effect of Sulfur Dioxide and Suspended Sulfates on Acute Respiratory Disease. Arch. Environ. Health 27: 129, 1973.

3. Levy, D., M. Gent, M. T. Newhouse. Relationship Between Acute Respiratory Illness and Air Pollution Levels in an Industrial City. American Review of Respiratory Disease. 116: 167, 1977.

4. Dungworth, D. L., W. L. Castleman, C. K. Chow, P. W. Mellick, M. G. Mustafa, B. Tarkington, and W. S. Tyler. Effect of Ambient Levels of Ozone on Monkeys. Fed. Proc. 34: 1670, 1975.

5. Schwartz, L. W., D. L. Dungworth, M. G. Mustafa, B. K. Tarkington, and W. S. Tyler. Pulmonary Responses of Rats to Ambient Levels of Ozone: Effects of 7-day Intermittent or Continuous Exposure. Lab. Invest. 34: 565, 1976.

6. King, T. P. Chemical and Biological Properties of Some Atopic Allergens. In Kunkel, H. G. and Dixon, F J. (eds): Advances in Immunology. New York, Academic Press, 1976. Vol. 23, pp. 77-105.

7. Osebold, J. W., S. L. Owens, Y. C. Zee, W. M. Dotson, and D. D. LaBarre. Immunological Alterations in the Lungs of Mice Following Ozone Inhalation. Arch. Environ. Health. 34: 258, 1979.

8. Plopper, C. G., C. K. Chow, D. L. Dungworth, M. Brummer, T. J. Nemeth. Effect of Low Level of Ozone on Rat Lungs. Exp. Molec. Path. 29: 400, 1978.

9. Ishizaka, K.: Cellular Events in the IgE Antibody Response. In Kunkel, H. G. and Dixon, F. J. (eds): Advances in Immunology. New York, Academic Press, 1976. Vol. 23, pp. 1-75.

10. Tada, T. and K. Ishizaka. Distribution of E-forming Cells in Lymphoid Tissues of the Human and Monkey. J. Immunol. 104: 377, 1970

11. Chang, D. P., B. K. Tarkington, and T. R. Duvall. Generation of Nuclei-mode Sulfuric Acid Aerosols for Health Effect Studies. J. Air Pollution Control Assoc. 28: 1137, 1978.

12. Kilborne, E. D. The Influenza Viruses and Influenza. Academic Press, New York, 1975.

13. Coulson, E. J. Systemic Anaphylaxis. In Williams, C. A. and Chase, M. W. (eds): Methods in Immunology and Immunochemistry. New York, Academic Press, 1976, Vol. 5, pp. 1-16.

14. Lang, G. M., W. Y. Lee, B. G. Carter, A. H. Sehon A. H. Communications: Production of Goat, Rat and Guinea Pig Antisera to Murine IgE. J. Immun. 116: 881, 1976.

15. Goldman, M.: Fluorescent Antibody Methods. Academic Press, New York, 1968.

16. Crowle, A. J.: Immunodiffusion. Academic Press, New York, 1973.

17. Braga, F., I. Mota. Homologous Passive Cutaneous Anaphylaxis (PCA) in Mice and Heterologous PCA Induced in Rats with Mouse IgE. Immunology. 30: 655, 1976.

18. Choppin, P. W. Replication of Influenza Virus in a Continuous Cell Line: High Yield of Infective Virus from Cells Inoculated at High Multiplicity. Virology. 38: 130, 1969.

19. Ibrahim, A. L., Y. C. Zee, and J. W. Osebold. The Effects of Ozone on the Respiratory Epithelium and Alveolar Macrophages of Mice. I. Interferon Production. Proc. Soc. Exp. Biol. Med. 152: 483, 1976.

20. Baublis, J. V., and G. C. Brown. Specific Response of the Immunoglobulins to Rubella Infection. Proc. Soc. Exp. Biol. Med. 128: 206, 1968.

REPRINTS AND MANUSCRIPTS

1. Osebold, J. W., S. L. Owens, Y. C. Zee, W. M. Dotson, and D. D. LaBarre. Immunological Alterations in the Lungs of Mice Following Ozone Inhalation. Arch. Environ. Health. 34: 258, 1979.

2. Osebold, J. W., L. J. Gershwin, and Y. C. Zee. Studies on the Enhancement of Allergic Lung Sensitization by Inhalation of Ozone and Sulfuric Acid Aerosol. J. Environ. Path. Tox. 3: 221, 1980.

3. Gershwin, L. J., J. W. Osebold, and Y. C. Zee. Immunologic Collagen in Induced Ascitic Fluid: Concurrent Preparation of Anti-Murine IgE. Am. J. Vet. Res., In Press.

4. Gershwin, L. J., J. W. Osebold, and Y. C. Zee. Immunoglobulin E-Containing Cells in Mouse Lung Following Allergen Inhalation and Ozone Exposure. Int. Archs. Allergy and Appl. Immun., In Press.

5. Yilma, T., Y. C. Zee, and J. W. Osebold. Immunofluorescence Determination of the Pathogenesis of Infection with Influenza Virus in Mice Following Exposure to Aerosolized Virus. J. Infect. Dis. 139: 458, 1979.

6. Owens, S. L., J. W. Osebold, and Y. C. Zee. Dynamics of B-Lymphocytes in the Lungs of Mice Exposed to Aerosolized Influenza Virus. Infect. and Immun., In Press.

7. Zee, Y. C., J. W. Osebold, and W. M. Dotson. Antibody Responses and Interferon Titers in the Respiratory Tracts of Mice After Aerosolized Exposure to Influenza Virus. Infect. and Immun. 25: 202, 1979.

8. Bolton, D. C., B. K. Tarkington, Y. C. Zee, and J. W. Osebold. An *In Vitro* System for Studying the Effects of Ozone on Mammalian Cell Cultures and Virses. Submitted for Publication.

9. Bolton, D. C., Y. C. Zee, and J. W. Osebold. The Biological Effects of Ozone on Representative Members of Five Groups of Animal Viruses. Submitted for Publication.

10. Ibrahim, A. L., Y. C. Zee, and J. W. Osebold. The Effects of Ozone on the Respiratory Epithelium and Alveolar Macrophages of Mice. I. Interferon Production. Proc. Soc. Exp. Biol. Med. 152: 483, 1976.

11. Ibrahim, A. L., Y. C. Zee, and J. W. Osebold. The Effects of Ozone on the Respiratory Epithelium of Mice. II. Ultrastructural Alterations. J. Environ. Path. Tox. 3: 251, 1980.

12. Zitnik, L. A., L. W. Schwartz, N. K. McQuillen, Y. C. Zee, and J. W. Osebold. Pulmonary Changes Induced by Low-Level Ozone: Morphological Observations. J. Environ. Path. Tox. 1: 365, 1978.